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(54) Title: ANTIBODY FRAGMENT-TARGETED IMMUNOLIPOSOMES FOR SYSTEMIC GENE DELIVERY

(57) Abstract: Nucleic acid-immunoliposome compositions useful as therapeutic agents are disclosed. These compositions preferably comprise (i) cationic liposomes, (ii) a single chain antibody fragment which binds to a transferrin receptor, and (iii) a nucleic acid encoding a wild type p53. These compositions target cells which express transferrin receptors, e.g., cancer cells. These compositions can be used therapeutically to treat persons or animals who have cancer, e.g., head and neck cancer, breast cancer or prostate cancer.

TITLE OF THE INVENTION

ANTIBODY FRAGMENT-TARGETED IMMUNOLIPOSOMES FOR SYSTEMIC GENE DELIVERY

BACKGROUND OF THE INVENTION

This invention provides methods for the preparation of antibody fragment-targeted liposomes ("immunoliposomes"), including lipid-tagged antibody fragment-targeted liposomes, methods for *in vitro* transfection using the immunoliposomes, and methods for systemic gene delivery *in vivo*. The liposomes of the present invention are useful for carrying out targeted gene delivery and efficient gene expression after systemic administration. The specificity of the delivery system is derived from the targeting antibody fragments.

An ideal therapeutic for cancer would be one that selectively targets a cellular pathway responsible for the tumor phenotype and which is nontoxic to normal cells. While cancer treatments involving gene therapy have substantial promise, there are many issues that need to be addressed before this promise can be realized. Perhaps foremost among the issues associated with macromolecular treatments is the efficient delivery of the therapeutic molecules to the site(s) in the body where they are needed. A variety of delivery systems (a.k.a. "vectors") have been tried including viruses and liposomes. The ideal delivery vehicle would be one that could be systemically (as opposed to locally) administered and which would thereafter selectively target tumor cells wherever they occur in the body.

The infectivity that makes viruses attractive as delivery vectors also poses their greatest drawback. Consequently, a significant amount of attention has been directed towards non-viral vectors for the delivery of molecular therapeutics. The liposome approach offers a number of advantages over viral methodologies for gene delivery. Most significantly, since liposomes are not infectious agents capable of self-replication, they pose no risk of transmission to other individuals. Targeting cancer cells via liposomes can be achieved by modifying the liposomes so that they selectively deliver their contents to tumor cells. There now exists a significant knowledge base regarding specific molecules that reside on the exterior surfaces of certain cancer cells. Such cell surface molecules can be used to target liposomes to tumor cells, because the molecules that reside upon the exterior of tumor cells differ from those on normal cells.

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference.

Current somatic gene therapy approaches employ either viral or non-viral vector systems. Many viral vectors allow high gene transfer efficiency but are deficient in certain areas (Ledley FD, et al. *Hum. Gene Ther.* (1995) **6**:1129-1144). Non-viral gene transfer vectors circumvent some of the problems associated with using viral vectors. Progress has been made toward developing non-viral, pharmaceutical formulations of genes for *in vivo* human therapy, particularly cationic liposome-mediated gene transfer systems (Massing U, et al., *Int. J. Clin. Pharmacol. Ther.* (1997) **35**:87-90). Features of cationic liposomes that make them versatile and attractive for DNA delivery include: simplicity of preparation; the ability to complex large amounts of DNA; versatility in use with any type and size of DNA or RNA; the ability to transfect many different types of cells, including non-dividing cells; and lack of immunogenicity or biohazardous activity (Felgner PL, et al., *Ann. NY Acad. Sci.* (1995) **772**:126-139; Lewis JG, et al., *Proc. Natl. Acad. Sci. USA* (1996) **93**:3176-3181). More importantly from the perspective of human cancer therapy, cationic liposomes have been proven to be safe and efficient for *in vivo* gene delivery (Aoki K et al., *Cancer Res.* (1997) **55**:3810-3816; Thierry AR, *Proc. Natl. Acad. Sci. USA* (1997) **92**:9742-9746). More than thirty clinical trials are now underway using cationic liposomes for gene therapy (Zhang W et al., *Adv. Pharmacology* (1997) **32**:289-333; RAC Committee Report: Human Gene Therapy Protocols-December 1998), and liposomes for delivery of small molecule therapeutics (e.g., antifungal and conventional chemotherapeutic agents) are already on the market (Allen TM, et al., *Drugs* (1997) **54 Suppl 4**:8-14).

The transfection efficiency of cationic liposomes can be dramatically increased when they bear a ligand recognized by a cell surface receptor. Receptor-mediated endocytosis represents a highly efficient internalization pathway present in eukaryotic surface (Cristiano RJ, et al., *Cancer Gene Ther.* (1996) **3**:49-57, Cheng PW, *Hum. Gene Ther.* (1996) 7:275-282). The presence of a ligand on a liposome facilitates the entry of DNA into cells through initial binding of ligand by its receptor on the cell surface followed by internalization of the bound complex. A variety of ligands have been examined for their liposome-targeting ability, including transferrin and folate (Lee RJ, et al., *J. Biol. Chem.* (1996) **271**:8481-8487). Transferrin receptors (TfR) levels are elevated in various types of cancer cells including prostate cancers, even those prostate cell lines derived from human lymph node and bone metastases (Keer HN et al., *J. Urol.* (1990) **143**:381-385); Chackal-Roy M et al., *J. Clin. Invest.* (1989) 84:43-50; Rossi MC, et al., *Proc. Natl. Acad. Sci. USA* (1992) **89**:6197-6201; Grayhack JT, et al., *J. Urol.* (1979) **121**:295-299).

Elevated TfR levels also correlate with the aggressive or proliferative ability of tumor cells (Elliot RL, et al., *Ann. NY Acad. Sci.* (1993) **698**:159-166). Therefore, TfR levels are considered to be useful as a prognostic tumor marker, and TfR is a potential target for drug delivery in the therapy of malignant cells (Miyamoto T, et al., *Int. J. Oral Maxillofac. Surg.* (1994) **23**:430-433:, Thorstensen K, et al., *Scand. J. Clin. Lab. Invest. Suppl.* (1993) **215**:113-120). In our laboratory, we have prepared transferrin-complexed cationic liposomes with tumor cell transfection efficiencies in SCCHN of 60%-70%, as compared to only 5-20% by cationic liposomes without ligand (Xu L, et al., *Hum. Gene Ther.* (1997) **8**:467-475).

In addition to the use of ligands that are recognized by receptors on tumor cells, specific antibodies can also be attached to the liposome surface (Allen TM et al., (1995) Stealth Liposomes, pp. 233-244) enabling them to be directed to specific tumor surface antigens (including but not limited to receptors) (Allen TM, *Biochim. Biophys. Acta* (1995) **1237**:99-108). These "immunoliposomes," especially the sterically stabilized immunoliposomes, can deliver therapeutic drugs to a specific target cell population (Allen TM, et al., (1995) Stealth Liposomes, pp. 233-244). Park, et al. (Park JW, et al., *Proc. Natl. Acad. Sci. USA* (1995) **92**:1327-1331) found that anti-HER-2 monoclonal antibody (Mab) Fab fragments conjugated to liposomes could bind specifically to HER-2 overexpressing breast cancer cell line SK-BR-3. The immunoliposomes were found to be internalized efficiently by receptor-mediated endocytosis via the coated pit pathway and also possibly by membrane fusion. Moreover, the anchoring of anti-HER-2 Fab fragments enhanced their inhibitory effects. Doxorubicin-loaded anti-HER-2 immunoliposomes also showed significant and specific cytotoxicity against target cells *in vitro* and *in vivo* (Park JW, et al., *Proc. Natl. Acad. Sci. USA* (1995) **92**:1327-1331). In addition, Suzuki et al., (Suzuki S, et al., *Br. J. Cancer* (1997) **76**:83-89) used an anti-transferrin receptor monoclonal antibody conjugated immunoliposome to deliver doxorubicin more effectively in human leukemia cells *in vitro*. Huwyler et al. (Huwyler J, et al., *Proc. Natl. Acad. Sci. USA* (1996) **93**:14164-14169) used anti-TfR monoclonal antibody immunoliposome to deliver daunomycin to rat glioma (RT2) cells *in vivo*. This PEGylated immunoliposome resulted in a lower concentration of the drug in normal tissues and organs. These studies demonstrated the utility of immunoliposomes for tumor-targeting drug delivery. It should be noted that the immunoliposome complexes used by Suzuki et al. and Huwyler et al. differ from those of the

invention described herein in that they are anionic liposomes and that the methods used by Suzuki et al. and Huwyler et al. are not capable of delivering nucleic acids.

Single-chain antibody fragments

Progress in biotechnology has allowed the derivation of specific recognition domains from Mab (Poon RY, (1997) Biotechnology International: International Developments in the Biotechnology Industry, pp. 113-128). The recombination of the variable regions of heavy and light chains and their integration into a single polypeptide provides the possibility of employing single-chain antibody derivatives (designated scFv) for targeting purposes. Retroviral vectors engineered to display scFv directed against carcinoembryonic antigen, HER-2, CD34, melanoma associated antigen and transferrin receptor have been developed (Jiang A, et al., *J. Virol.* (1998) **72**:10148-10156, Konishi H, et al., *Hum. Gene Ther.* (1994) **9**:235-248:, Martin F, et al., *Hum. Gene Ther.* (1998) **9**:737-746). These scFv directed viruses have been shown to target, bind to and infect specifically the cell types expressing the particular antigen. Moreover, at least in the case of the carcinoembryonic antigen, scFv was shown to have the same cellular specificity as the parental antibody (Nicholson IC, *Mol. Immunol.* (1997) **34**:1157-1165).

The combination of cationic liposome-gene transfer and immunoliposome techniques appears to be a promising system for targeted gene delivery.

SUMMARY OF THE INVENTION

We constructed a variety of immunoliposomes that are capable of tumor-targeted, systemic delivery of nucleic acids for use in human gene therapy. Based upon the data given in the Examples below these immunoliposome-DNA complexes incorporating the TfRscFv are capable of producing a much higher level of transfection efficiency than the same liposome-DNA complex bearing the complete Tf molecule. Therefore, in one aspect of the invention the immunoliposomes of the invention can be used to produce a kit for high efficiency transfection of various mammalian cell types that express the transferrin receptor. In one aspect of the invention, we constructed an scFv protein with a lipid tag such that the lipid is added naturally by the bacterial cell to allow easy incorporation of the scFv into liposomes while also avoiding chemical reactions which can inactivate the scFv.

The lipid-tagged scFv-immunoliposomes are prepared basically by two methods: a lipid-film solubilization method and a direct anchoring method. The lipid-film solubilization

method is modified from the detergent dialysis method, which was described by Laukkanen ML, et al., (Laukkanen ML, et al., *Biochemistry* (1994) **33**:11664-11670) and de Kruif et al., (de Kruif et al., *FEBS Lett.* (1996) **399**:232-236) for neutral or anionic liposomes, with the methods of both hereby incorporated by reference. This method is suitable for attaching lipid-tagged scFv to cationic liposomes as well. In the lipid-film solubilization method, the lipids in chloroform are evaporated under reduced pressure to obtain a dry lipid film in a glass round-bottom flask. The lipid film is then solubilized with 0.5-4%, preferably 1%, n-octyl β-D-glucoside (OG) containing the lipid-modified scFv and vortexed. After dilution with sterile water, the solution is briefly sonicated to clarity.

The second method for attaching lipid-tagged antibodies or antibody fragments is the direct anchoring method that is specifically useful for attaching the *E. coli* lipoprotein N-terminal 9 amino acids to an scFv (lpp-scFv) or other lipid-modified antibody or fragments and attaching these to preformed liposomes. For attaching the scFv to preformed liposomes, the lipid-modified scFv in 1% OG is added to preformed liposomes while vortexing, at volume ratios from 1:3 to 1:10. The mixture is vortexed for approximately a further 5-10 minutes to obtain a clear solution of scFv-immunoliposomes. The remaining OG and the uncomplexed scFv can be eliminated by chromatography, although they will not interfere very much with the subsequent usage. Separation experiments, i.e., ultrafiltration with Centricon-100 (Amicon), Ficoll-400 floatation (Shen DF, et al., *Biochim. Biophys. Acta* (1982) **689**: 31-37), or Sepharose CL-4B (Pharmacia) chromatography, demonstrated that virtually all the lipid-tagged scFv molecules added have been attached or anchored to the cationic liposomes. This is an improvement over the much lower attachment rate of lpp-scFv to neutral or anionic liposomes. Therefore, this improvement makes it unnecessary to include a further purification step to remove the unattached scFv.

Any antibodies, antibody fragments, or other peptide/protein ligands that can be modified to have one or more lipid-tags on the surface are useful in the present invention. Other lipid-modification methods include directly conjugating a lipid chain to an antibody or fragment, as described in Liposome Technology, 2nd Ed., Gregoriadis, G., Ed., CRC Press, Boca Raton, FL, 1992.

In another aspect of the invention a cysteine was added at the C-terminus of the scFv sequence and the protein was expressed in the inclusion bodies of *E. coli*, then refolded to produce active scFv. The C-terminal cysteine provided a free sulfhydryl group to facilitate the

conjugation of the scFv to liposomes. There are two strategies which can be used in the conjugation process. 1) Pre-linking method: The first step is to conjugate the scFv-SH with the cationic liposome which contains a maleimidyl group or other sulfhydryl-reacting group, to make the scFv-liposome. The nucleic acids are then added to the scFv-liposome to form the scFv-liposome-DNA complex. The pre-linking is designated since scFv is linked before DNA complexing. 2) Post-linking method: This strategy is to complex the cationic liposome with nucleic acids first to form a condensed structure. The scFv-SH is then linked onto the surface of DNA-liposome complex to produce scFv-liposome-DNA. The post-linking is designated since scFv is linked after DNA complexing. The post-linking strategy ensures that 100% of scFv linked are on the surface of the complex, accessible to receptor binding. Therefore, this method can make a better use of the targeting ligand scFv and a better controlled inside structure of the complex.

The nucleic acid-immunoliposome complexes, regardless of whether the antibody or antibody fragment is lipid tagged or conjugated to the liposome, can be used therapeutically. Preferably the complexes are targeted to a site of interest, preferably to a cell which is a cancer cell, more preferably to a cell expressing a transferrin receptor. The targeting agent is the antibody or antibody fragment which preferably binds to a transferrin receptor. The nucleic acid is the therapeutic agent and is preferably a DNA molecule and more preferably encodes a wild type p53 molecule. The nucleic acid-immunoliposome complexes, preferably in a therapeutic composition, can be administered systemically, preferably intravenously.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 show scFv TfR lipid-tag construction.

Figure 2 shows a Western blot analysis of scFv-liposome-targeted p53 expression *in vivo* in tumor xenografts with systemic administration.

Figure 3 shows pCMVp53 and pCMVpRO constructs.

Figure 4 shows p53-3'Ad construction.

Figure 5 shows construction of scFvTfR-cysteine with a His tag.

Figure 6 shows construction of scFvTfR-cysteine without a His tag.

Figure 7 shows construction of scFvTfR-cysteine with a cellulose binding domain (CBD) tag and with an S-tag.

Figure 8 shows a Coomassie Blue stained SDS-polyacrylamide gel of purified TfRscFv protein produced by the conjugation method.

Figure 9 shows a Western blot analysis of conjugation method produced TfRscFv-liposome-targeted p53 expression *in vivo* in tumor xenografts with systemic administration.

DETAILED DESCRIPTION OF THE INVENTION

The invention is directed to immunoliposomes and methods of making and using these immunoliposomes. A variety of embodiments are disclosed including immunoliposomes with different tags and various methods with which to attach the scFv to the liposomes. The immunoliposomes may include lipid tags or be linked through a reducing group, which in a preferred embodiment is a free sulfhydryl.

Mutant forms of the tumor suppressor gene p53 have been associated with more than 50% of human cancers, including 15-50% of breast and 25-70% of metastatic prostate cancers. Abnormalities in p53 also correlate with poor prognosis in various types of malignancies. Therefore, the capability to systemically deliver and target gene therapy specifically to tumors to efficiently restore wtp53 function will be an important therapeutic modality in cancer treatment. Thus the immunoliposomes produced by the method of this invention will be useful as an effective new cancer therapeutic modality not just for restoration of wtp53 function but also as a tumor targeted systemic delivery vehicle for other therapeutic genes.

The invention is illustrated by the following Examples.

Example 1

Construction and Expression of Biosynthetically Lipid-tagged scFv

1. Construction of the Expression Vector for TfRscFv

To construct the expression vector, we used the vector pLP1 which contains an amino acid linker sequence between the *E. coli* lipoprotein signal peptide (ssLPP) and the scFv cloning site (de Kruif et al., *FEBS Lett.* (1996) **399**:232-236). This vector contains both c-myc and His_6 tag sequences that can be used for purification and detection of the expressed scFv (Figure 1).

We obtained a plasmid expression vector, pDFH2T-vecOK, which contains the single chain fragment for the 5E9 (Haynes et al., *J. Immunol.* (1981) **127**:347-351) antibody linked to a DNA binding protein, which recognizes the human transferrin receptor (TfR). This vector also

contains the sequence for a DNA binding protein, and there are no unique restriction enzyme sites flanking the scFv sequence in pDFH2T-vecOK. Therefore, we cloned the VH-linker-Vκ scFv by PCR amplification of the desired fragment using a 5' primer (5' GGCCCATGGAGGTGCAGCTGGTGG 3' (SEQ ID NO:1)) (RB551) containing an *Nco*I site and a 3' primer (RB552) (5' CCGGAATTCGCGGCCGCTTTTATCTCCAGCTTGGTC 3' (SEQ ID NO:2) containing a *Not*I site. The PCR amplification using primers RB551 and RB552 amplified the scFv for TfR from pDFH2T-vecOK from the Met at base 81 to Lys at base 821. The pLP1 vector also contains sequences for the *E. coli* lipoprotein signal peptide (ssLPP) and the *E. coli* lipoprotein N-terminal 9 amino acids (LPP), as described by Laukkanen ML, et al., (Laukkanen ML, et al., *Biochemistry* (1994) **33**:11664-11670) and de Kruif et al (de Kruif et al., *FEBS Lett.* (1996) **399**:232-236). The insertion of these sequences will lead to fatty acid acylation of the expressed signal in the *E. coli* host and its insertion into the bacterial membrane. The vector also has a non-critical 10 amino acid linker sequence to increase the space between the lipid-tag site and the scFv. Purification of the lipid modified scFv sequence from the bacterial membrane results in an active molecule that can be attached or inserted into liposomes.

2. Expression and Purification of the TfRscFv

We transformed *E. coli* expression host SF110 F' with the expression vector constructed above. While the host cell is not critical it is preferred that it contain expressed lac repressor. A number of clones were selected and the one that produces the best yield of scFv was chosen. The lipid-modified scFv (lpp-scFv) was isolated from the bacterial membrane using Triton X-100 as described by de Kruif et al., (de Kruif et al., *FEBS Lett.* (1996) **399**:232-236). For purification a single colony was resuspended in 200 µl LB containing 5% glucose and the appropriate antibiotics. The mixture was plated onto two 90 mm LB agar plates containing 5% glucose and the appropriate antibiotics and grown overnight. The next day, the cells were washed from the plates and used to inoculate a total of 5 liters of LB containing 0.1% glucose and the appropriate antibiotics. The cultures were grown at 25 °C, at 200 rpm for 6 hours until the OD_{600} reached 0.5 to 0.7. IPTG was added to a final concentration of 1 mM and the cultures were further incubated overnight. The next day, the bacterial cultures were collected by centrifugation and lysed in 200 ml lysis buffer at room temperature for 30 minutes. The sample was sonicated at 28 watts for 5 minutes with cooling on ice. The lysis buffer contains 20 mM HEPES pH 7.4 to 7.9, 0.5 M

NaCl, 10% glycerol, and 0.1 mM PMSF. The only deviations from the cited protocol include washing and elution of metal affinity columns in buffer containing 20 mM HEPES pH 7.4 to 7.9, 0.5 M NaCl, 10% glycerol, 0.1 mM PMSF, 1% n-octyl β-D-glucoside (OG), and 10% glycerol containing 20 and 200 mM imidazole, respectively. The eluted samples of lpp-scFv were analyzed by SDS-PAGE and Western Blot using anti-c-myc antibody 9E10 which confirmed that the purified scFv showed a band of the size of about 30 kDa.

Example 2

Preparation of Lipid-tagged scFv-immunoliposomes by a Lipid-film Solubilization Method

This example discloses a detailed procedure of lipid-film solubilization method to prepare lipid-tagged scFv-immunoliposomes. 5 μmol lipids (DOTAP/DOPE, 1:1 molar ratio) in chloroform are evaporated under reduced pressure to obtain a dry lipid film in a glass round-bottom flask. To the lipid film is added 0.5 ml 1% OG, 20 mM HEPES, 150 mM NaCl, pH 7.4, containing the lipid-modified scFv. This is incubated 10-20 minutes at room temperature and then vortexed to solubilize the lipid membrane. 2 ml sterile water is then added to dilute the scFv-lipid mixture. The solution is briefly sonicated to clarity in a bath-type sonicator at 20°C. The scFv-liposome is a clear solution with a limited amount of detergent OG left. The OG and the uncomplexed scFv can be eliminated by chromatography with Sepharose CL-4B or Sephacryl S500, even though they do not interfere a lot with the subsequent use.

Example 3

Preparation of Lipid-tagged scFv-immunoliposomes by a Direct Anchoring Method

This example provides a direct anchoring method to prepare lipid-tagged scFv-immunoliposomes. 20 μmol lipids (LipA-H, see below for compositions and ratios) prepared as dry lipid film in a glass round-bottom flask is added to 10 ml pure water and sonicated in a bath-type sonicator for 10-30 min at room temperature (LipA, B, C) or at 65°C (LipD, E, G, H, or any composition with Cholesterol (Chol)). The cationic liposomes prepared are clear solutions, their compositions and ratios are as follows:

LipA	DOTAP/DOPE	1:1 molar ratio
LipB	DDAB/DOPE	1:1 molar ratio
LipC	DDAB/DOPE	1:2 molar ratio
LipD	DOTAP/Chol	1:1 molar ratio
LipE	DDAB/Chol	1:1 molar ratio
LipG	DOTAP/DOPE/Chol	2:1:1 molar ratio
LipH	DDAB/DOPE/Chol	2:1:1 molar ratio

For attaching the scFv to preformed liposomes, the lipid-modified scFv (lpp-scFv) in 20 mM HEPES, 150 mM NaCl, pH 7.4, containing 1% OG is added to preformed liposomes while vortexing, at volume ratios from 1:3 to 1:10. The mixture is vortexed for a further 1 to 5 min to get a clear solution of scFv-immunoliposomes. The remaining OG and the uncomplexed scFv can be eliminated by chromatography, although they do not interfere very much with the subsequent usage. Separation experiments, i.e., ultrafiltration with Centricon-100 (Amicon), Ficoll-400 floatation (Shen DF, et al., *Biochim Biophys Acta* (1982) **689**:31-37), or Sepharose CL-4B (Pharmacia) chromatography, demonstrated that virtually all the lipid-tagged scFv added have been attached or anchored to the cationic liposomes. This is in contrast to the much lower attachment rate of lpp-scFv to neutral or anionic liposomes. Therefore, it is unnecessary to have a further purification step to get rid of the unattached scFv.

Example 4

Immunoreactivity of Lipid-tagged scFv-immunoliposomes Revealed by ELISA, FACS and Immunofluorescence

This example provides the characterization of the anti-TfR scFv-immunoliposomes with respect to their ability of binding to the TfR(+) cells. The human prostate cancer cell line DU145 and the human squamous cell carcinoma of head and neck cell line JSQ-3 served as the TfR+ target cells for these studies.

Indirect cellular enzyme-linked immunosorbent assay (ELISA) was employed to determine the immunoreactivity of the lpp-scFv before and after attachment to liposomes. Confluent JSQ-3 cells in 96-well plates were fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature. The plate was blocked with 5% fetal bovine serum (FBS) in PBS at 30°C

for 30 min. The lpp-scFv, scFv-immunoliposomes and liposomes were added to wells in duplicate and incubated at 4°C overnight. After three PBS-washes, an anti-c-myc monoclonal antibody was added to each well in 3% FBS in PBS and incubated at 37°C for 60 min. After three PBS-washes, HRP-labeled goat-anti-mouse IgG (Sigma) diluted in 3% FBS was added to each well and incubated for 30 min at 37°C. The plate was washed three times with PBS and 100 µl substrate 0.4 mg/ml OPD in citrate phosphate buffer (Sigma) was added to each well. The color-development was stopped by adding 100 µl 2 M sulfuric acid to each well. The plate was read by an ELISA plate reader (Molecular Devices Corp.) at 490 nm. Indirect cellular ELISA demonstrated that the anti-TfR scFv retained its immunoreactivity after incorporation into the liposome complex (Table 1).

Table 1

Binding of anti-TfR scFv-liposomes to JSQ-3 cells*

Lip(A) only	0.142 ± 0.036
scFv-LipA1	1.134 ± 0.038
scFv-LipA2	1.386 ± 0.004
lpp-scFv	0.766 ± 0.009

* ELISA, OD_{490}, Mean ± SD

scFv-LipA1: by lipid-film solubilization method.

scFv-LipA2: by direct anchoring method.

For FACS analysis, anti-TfR scFv-Lip(A), was incubated at 4°C with JSQ-3 and DU145 cells, then with FITC-labeled sheep anti-mouse IgG, also at 4°C. Incubation of JSQ-3 cells with the scFv-Lip(A) resulted in a fluorescence shift identical to that observed with the unattached free anti-TfR lpp-scFv antibody, demonstrating a significant amount of binding to the target cells. In contrast, the untargeted liposome demonstrated very low binding to the cells. Similar results were observed with prostate tumor cell line DU145. Here also, the scFv-Lip(A) complex demonstrated clear, substantial binding to the tumor cells, as compared to the untargeted Lip(A). The FACS data is summarized in Table 2, where the fluorescence shift is indicated as the percent of the cells displaying fluorescence above the threshold level (percent of positive cells). In these studies also, the level of binding to the cells, represented by the percent of positive cells, was

similar to that of the unattached free scFv further indicating that incorporation into the liposome complex did not inactivate the immunological activity of the anti-TfR lpp-scFv. It should be noted that the liposome preparation used for these initial experiments with DU145 was that optimized for JSQ-3 cells. Therefore, the binding of the scFv-targeted liposome complex to the prostate tumor cells can be further enhanced by the use of the liposome complex optimized for this cell type.

Table 2

FACS Analysis of TfRscFv-liposome Binding to JSQ-3 and DU145

	JSQ-3		DU145	
Transfected by	% Positive	Mean[a]	% Positive	Mean[a]
Untransfected	3.46	4.07	2.22	3.40
Lip(A)	9.69	6.26	4.51	4.07
scFv-LipA1	86.38	19.8	50.19	12.40
scFv-LipA2	89.58	21.30	39.52	11.1
Free lpp-scFv	85.09	21.30	78.09	18.40
HB21[b]	99.44	69.80	98.70	64.90

a: Mean of the relative fluorescence

b: Parental monoclonal antibody of the anti-TfR scFv

Indirect immunofluorescence staining with scFv-liposome (where Lip(A) had been labeled with rhodamine-DOPE) and FITC-labeled anti-mouse IgG following anti-c-myc antibody, confirmed the binding of the scFv-targeted liposome complex to the JSQ-3 cells. The concurrence of the red and green fluorescence in the transfected cells demonstrates that the anti-TfR scFv (indicated by the FITC-labeled anti-c-myc antibody as green fluorescence) does indeed direct the rhodamine-labeled Lip(A) to the cells. Moreover, the high level of cellular binding of the scFv-Lip(A) system is demonstrated by the large percentage of red/green double-positive fluorescent cells.

Example 5

Optimization of scFv-immunoliposome

Mediated Gene Transfection of Target Cells *In Vitro*

We determined the *in vitro* transfection efficiency of the anti-TfR scFv-Lip(A) complex in JSQ-3 cells using β-galactosidase as the reporter gene. In these studies the reporter construct used contained the β-galactosidase gene under the control of the CMV promoter (pCMVb), the same promoter used in pCMVp53 (Figure 3). The level of β-Gal expression in the transfected cells (correlating with the transfection efficiency) was assessed by β-Gal enzymatic assay (Xu L, et al., *Hum. Gene Ther.* (1997) **8**:467-475). As shown in Table 3, the attachment of the anti-TfR scFv to the Lip(A) resulted in a doubling of the enzyme activity in the scFv-Lip(A)-pCMVb transfected cells, as compared to the untargeted liposome complex. This level of expression was also found to be virtually identical to that observed when transferrin itself was used as the targeting ligand (Tf-Lip(A)-pCMVb). Moreover, this increase in gene expression was shown to be reporter gene DNA dose dependent. Table 4 shows the optimization of scFv-liposome mediated transfection of JSQ-3 cells.

Table 3

Transfection of JSQ-3 Cells by Anti-TfR scFv-liposomes*

DNA (μg/well)	Lip(A) only	Tf-Lip(A)	scFV-LipA1	scFv-LipA2
1.0	475	1031	997	1221
0.5	601	981	811	854
0.25	266	503	578	471
0.125	130	262	215	236

* milliunits/mg protein, β-galactosidase equivalent, β-Gal enzymatic assay

scFv-LipA1: by lipid-film solubilization method

scFv-LipA2: by direct anchoring method

Table 4

Optimization of scFv-liposome transfection to JSQ-3*

DNA/Lip (μg/nmol)	Lip(A) only	scFv-LipA1	scFv-LipA2	scFv-LipB	scFv-LipD	scFv-LipG
1/8	1.559	2.793	2.642	1.827	0.874	0648
1/10	1.776	2.846	2.83	2.268	1.606	1283
1/12	1.868	2.772	2.815	2.175	1.257	1416
1/14	1.451	3.031	2.797	2.31	1.78	1.656

* β-Gal enzymatic assay, OD_{405}

scFv-LipA1: by lipid-film solubilization method

scFv-LipA2: by direct anchoring method

Example 6

scFv-immunoliposome Mediated p53 Gene Transfection

Target to Tumor Cells Causing Sensitization to Chemotherapeutic Agents

1. Anti-TfR scFv Facilitated Liposome-Mediated wtp53 Gene Transfection *In Vitro*

The expression of exogenous wtp53 in JSQ-3 tumor cells transfected with the anti-TfR scFv-targeted Lip(A)-p53-3'Ad was assessed by co-transfection of an expression plasmid (pBP100) which contains the luciferase reporter gene under the control of a p53 responsive promoter (Chen L, et al., *Proc. Natl. Acad. Sci. USA* (1998) **95**:195-200). Consequently, the higher the level of exogenous wt p53 expression (representing the scFv-Lip(A)-p53-3'Ad transfection efficiency), the higher the level of luciferase activity. This luciferase enzyme activity is expressed as relative light units (RLU). As was demonstrated above with the β-gal reporter gene, the addition of the anti-TfR scFv as the targeting agent to the Lip(A)-p53-3'Ad complex resulted in a significant increase in transfection efficiency and wtp53 protein expression (as expressed by RLU of Luciferase activity) over the untargeted Lip(A)-p53-3'Ad complex (Table 5). Once again, the level of p53 expression in the scFv-Lip(A)-p53-3'Ad transfected cells was similar to that observed when transferrin itself was used as the targeting ligand (LipT(A)-p53-3'Ad). Therefore, these findings indicate that the anti-TfR single-chain antibody strategy is a useful method of targeting the cationic liposome complex, and delivering a biologically active wtp53 gene, to tumor cells.

Table 5

In Vitro p53 Expression Mediated by Different Liposomes in JSQ-3 cells

Transfected by		RLU*
Medium	+ p53-3'Ad + pBP100	158
Lip(A)	+ p53-3'Ad + pBP100	4073
LipT(A)	+ p53-3'Ad + pBP100	7566
scFv-Lip(A1)	+ p53-3'Ad + pBP100	6441

* Relative light units per well

2. Anti-TfR scFv-immunoliposome mediated p53 gene restoration sensitized the tumor cells to the cytotoxicity of Cisplatin (CDDP).

For the p53-induced apoptosis study, mouse melanoma cell line B16 was transfected with anti-TfR scFv-immunoliposome complexed with p53-3'Ad (Figure 4) or pCMVpRo plasmid (Figure 3) DNA (scFv-Lip(A)-p53 and scFv-Lip(A)-pRo, respectively) at a dose of 5 μg DNA/2 x 10^5 cells in 2 sets of 6-well plates. For comparison, transferrin-liposome-DNA (LipT-p53 or LipT-pRo) were also transfected at a dose of 5 μg DNA/2 x 10^5 cells. 24 hours later, CDDP was added to one set of plates to 10 μM final concentration. 24 and 48 hours after the drug was added, both the attached and floating cells were collected for apoptosis staining. The cells were stained with an Annexin V-FITC Kit (Trevigen, Inc., Gaithersburg, MD) according to manufacturer's protocol. Annexin V is a lipocortin, a naturally occurring blood protein and anti-coagulant. The stained cells were analyzed on a FACStar cytometer (Becton and Dickinson). Table 6 summarizes the results of the apoptosis analysis.

Table 6

Apoptosis of B16 Cells Induced by Liposomal p53-gene Restoration and CDDP*

	24 hours		48 hours	
Transfected by	- CDDP	+ CDDP	- CDDP	+ CDDP
Untransfected	0.22	4.4	6.33	20.11
LipA-p53	15.9	26.7	15.02	26.52
scFv-LipA-p53	13.9	38.4	34.94	43.7
scFv-LipA-pRo	8.1	19.9	24.14	37.59
Tf-LipA-p53	22.4	29.5	34.47	31.7
Tf-LipA-pRo	14.1	12.6	14.00	25.34

* % of apoptotic cells (Annexin V-FITC positive)

Without CDDP there was no increase in the percent of apoptotic cells induced at 24 hours by the addition of the scFv ligand as compared to the amount induced by the liposome complex alone. However, by 48 hours, there is a greater than 2-fold increase in the percent of apoptotic cells by the addition of the targeting scFv to the lipoplex. With CDDP there is a significant increase in apoptotic cells (approximately 1.5-fold) even at 24 hours as compared to the untargeted liposome complex. More significantly, this increase in apoptotic cells in combination with CDDP is more pronounced using the scFv to the Tf receptor as the targeting ligand than using the Tf molecule itself. This increase correlates with transfection efficiency.

Example 7

scFv-immunoliposome-targeted wtp53 Gene Delivery and Expression *In Vivo* with Systemic Administration

To examine the ability of the anti-TfR scFv containing liposomes to deliver wtp53 specifically to tumor tissue *in vivo*, scFv-Lip(A)-p53-3'Ad (Figure 4) or the untargeted Lip(A)-p53-3'Ad (Figure 4) was injected intravenously into nude mice bearing JSQ-3 subcutaneous xenograft tumors. Two days after injection, the tumors were excised and protein isolated from liver and skin, as well as the tumor, for Western blot analysis (Xu L, et al., *Hum. Gene Ther.* (1997) **8**:467-475). Equal amounts of protein (100 µg, as determined by concentration) were loaded in each lane. As shown in Figure 2, the tumor from the mouse systemically treated with the scFv-Lip(A)-p53-3'Ad complex, labeled scFv-Lip(A)-p53 in Figure 2, displayed a very intense p53 signal as well as the additional lower band indicative of a high level of expression of the exogenous wtp53, while only the lower expression of the endogenous mouse p53 is evident in both the skin and the liver. In contrast, as would be expected based upon our earlier results, a significantly lower level of exogenous p53 expression is evident in the tumor isolated from the untargeted Lip(A)-p53-3'Ad injected mouse, labeled Lip(A)-p53 in Figure 2. Therefore, the liposome complex targeted by our new and unique anti-TfR lpp-scFv ligand can clearly deliver exogenous genes selectively to the tumor *in vivo*. These results demonstrate the potential of this new way of efficiently targeting systemically delivered, cationic liposome complexes specifically to tumors *in vivo*.

Example 8

Construction and Purification of TfRscFv with a 3' Cysteine for Use in the Conjugation Method

In the absence of a lipid tag, another method was devised to attach the purified TfRscFv protein to the lipoplex. This approach entails the conjugation of the single chain protein to cationic liposomes via a reducible group such as a sulfhydryl group. In the preferred embodiment a cysteine residue is added at the 3' end of the TfRscFv protein. Reduction of this cysteine results in a free sulfhydryl group which is capable of being conjugated to cationic liposomes, thus targeting the lipoplex to cells expressing the transferrin receptor. While the following examples use cysteine as the reducible group it is obvious that other similar reducing groups would also work with this method.

1. Construction

A. Construction of an Expression Vector Containing a 3' Cysteine with a Histidine Tag for Use in the Conjugation Method of Producing TfRscFv Immunoliposomes

As in Example 1, the VH-linker-Vκ scFv for the TfR was obtained from plasmid expression vector, pDFH2T-vecOK (described in Example 1). Using a 5' primer (5' GGCCCATGGAGGTGC AGCTGGTGG 3' (SEQ ID NO:3)) for PCR amplification, an *Nco*I site was introduced into pDFH2T-vecOK. The nucleotide sequence for the cysteine residue as well as a *Not*I restriction site was introduced using a 3' primer (5' GGCGCGGCCGCGCATTTTATCTCCAGCTTG 3' (SEQ ID NO:4)). The PCR product was cloned into *Nco*I and *Not*I sites of the commercial vector pET26b(+) (Novagen). This vector also contains, 5' of the *Nco*I site, the pelB leader signal sequence. The presence of this sequence in the expression vector allows transport of the protein to the periplasmic space. To aid in purification of the protein, the pET26b(+) vector also contains a Histidine tag sequence 3' of the *Not*I site (Figure 5).

B. Construction of an Expression Vector Containing a 3' Cysteine without a Histidine Tag for Use in the Conjugation Method of Producing TfRscFv Immunoliposomes

For human use as a therapeutic delivery vehicle, it is preferable that the TfRscFv be produced without the Histidine tag. Therefore, the construct described in Example 8, section 1. A. was modified to eliminate this tag in the final protein product. To accomplish this, the same

5' primer as described above (in Example 8, section 1. A) was used. However, a different 3' primer was used. In addition to the nucleotide sequence for the cysteine residue and the *Not*I restriction site, this primer (5'GGCGCGGCCGCTCAGCATTTTATCTCCAGCTTG 3' (SEQ ID NO:5)), introduced a DNA stop codon adjacent to the cysteine sequence and before the *Not*I site (Figure 6). Thus, the protein product of this construct will not contain the His-tag.

C. Construction of an Expression Vector Containing a 3' Cysteine with a 5'CBD™-Tag for Use in the Conjugation Method of Producing TfRscFv Immunoliposomes

A third alternative construct containing a cysteine residue for linkage to the cationic lipoplex using the conjugation method was also made. For this construct (Figure 7), the same two primers described above in Example 8, section 1. B, were used. Thus no His-tag would be present in the protein product. However, the PCR product of these reactions was cloned into a different vector, pET37b(+) (Novagen). This vector contains a cellulose binding domain tag (CBD™-tag) and an S-tag, both 5' of the *Nco*I site in the vector. The CBD-tag sequence encodes a cellulose binding domain derived from a microbial cellulase. Thus, the presence of this tag enables the use of cellulose-based supports for highly specific, low cost affinity purification of the protein product. The presence of the S-tag present in this construct allows for easy detection of the protein product on Western blots and for easy enzymatic quantitation of protein amounts.

2. Purification of the TfRscFv containing the Cysteine Residue

The commercially available *E. coli* expression host BL21(DE3), which contains the expressed lac repressor, was transformed with an expression vector (all three were used individually) described above in Example 8, section 1. A number of clones were selected and the ones that produced the best yield of TfRscFv were chosen. Purification of the protein from the construct described above in Example 8, section 1. A, with the histidine tag is given in detail as an example, although the same method is used for purification of the cysteine containing TfRscFv protein from all three constructs described in Example 8, section 1. The majority of the TfRscFv protein (approximately 90%) was found not to be soluble but to be contained within the inclusion bodies. Therefore, the TfRscFv containing the cysteine-linker was purified from the inclusion bodies as follows. A single clone was inoculated into 5-10 ml LB containing 50 μg/ml Kanamycin, and grown at 37°C, and 250 rpm to an OD_{600} of 0.5–0.7 (4-5 hrs). 30 ml of the mini culture was pelleted, suspended in LB broth, added to 1 L LB containing 50 μg/ml Kanamycin

and incubated at 37°C and 250 rpm. to an OD_{600} of 0.5–0.7 (4-5 hrs). To induce expression of the TfRscFv protein, IPTG at a final concentration of 1 mM was added to the culture at this time and incubation continued for an additional 4 hrs. This time was determined to yield the maximum level of protein expression. The bacterial cultures were then collected by centrifugation and lysed in 100 ml of cold 20 mM Tris-HCl, pH 7.5, containing 100 µg/ml lysozyme. at 30°C for 15 minutes. The sample was sonicated at 10 watts for 5 minutes (in 30 second bursts) with cooling on ice. The inclusion bodies were isolated by centrifugation at 13.000 g for 15 minutes. The resulting pellet was washed three times in cold 20 mM Tris-HCl buffer. pH 7.5. The purity and quantity of the inclusion bodies were determined by SDS-polyacrylamide gel electrophoresis before solubilization.

The isolated inclusion bodies were dissolved in 100 mM Tris-HCl, pH 8.0 containing 6 M guanidine-HCl and 200 mM NaCl (6 M GuHCl buffer) and centrifuged at 12,300 g for 15 minutes to remove insoluble debris. 2-mercaptoethanol was added to the supernatant to a final concentration equal to approximately 50 molar fold of the protein concentration and the mixture incubated with rotation for 1 hour at room temperature. The presence of such a high concentration of guanidine-HCl and the reducing agent results in a totally unfolded protein. Refolding of the TfRscFv protein was accomplished by dialysis at 4°C against decreasing concentrations of guanidine-HCl in the absence of 2-mercaptoethanol. Dialysis was performed for 24 hours each against the following concentrations of guanidine-HCl in 100 mM Tris-HCl, pH 8.0 and 200 mM NaCl: 6 M, 3 M, 2 M, 1 M and 0.5 M. The last dialysis was against three changes of just 100 mM Tris-HCl, pH 8.0 and 200 mM NaCl. The fourth dialysis solution (of 1 M guanidine-HCl) also contained 2 mM glutathione (oxidized form) and 500 mM L-arginine. These reagents allow the partially refolded protein to form the proper disulfide bonds to produce the correct protein conformation. The solution was clarified by centrifugation at 13000 g to remove aggregates. The sample was concentrated approximately 1.5 fold using the Centrplus centrifugal filter (Amicon) at 3000 g for 90 min. SDS-PAGE showed a single band of the solubilized cysteine containing TfRscFv with a molecular weight of approximately 28-30 kDa containing only minor contaminants (Figure 8).

Example 9

Preparation of scFv-liposomes by the Conjugation Method

1. Reduction of scFv

The purified TfRscFv was reduced by DTT to obtain monomer scFv-SH as follows: To scFv in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) was added 1 M DTT to a final concentration of 1-50 mM. After rotation at room temperature for 5-10 min, the protein was desalted on a 10-DG column (Bio-Rad). The free -SH group was measured by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (G.L. Ellman (1959) *Arch. Biochem. Biophys.* **82**:70-77. P.W. Riddles, R.L. Blakeley, B. Zeruer (1993) *Methods Enzymol.* **91**:49-60) and calculated as -SH/protein molar ratio, or number of free -SH per scFv molecule (Table 7). The results indicate that 1-10 mM DTT is appropriate for the scFv reduction.

Table 7

Reduction of TfRscFv

DTT Concentration (mM)	-SH/scFv molar ratio
0	0.15
1	0.45
10	1.94
20	2.26
50	3.03

2. Liposome Preparation

4-(*p*-maleimidophenyl)butyrate-DOPE (MPB-DOPE) (Avanti Polar Lipids) is included in the seven liposome formulations described in Example 3, to a 5-8% molar of total lipids. The MPB-liposomes were prepared the same way as described in Example 3. Other liposome preparation methods can also be used to prepare the cationic liposomes. For example, the ethanol injection method modified from that described by Campbell MJ (*Biotechniques* 1995 Jun; **18(6)**:1027-32) was used successfully in the present invention. In brief, all lipids were solubilized in ethanol and mixed, injected into vortexing pure water of 50-60°C with a Hamilton syringe. The solution was vortexed for a further 10-15 min. The final concentration was 1-2 mM total lipids. The ethanol injection method is faster, easier and more robust. 1 M HEPES, pH 7.5 (pH 7.0-8.0) was added to a final concentration of 10-20 mM. Since we have found that the

maleimide group is not stable in aqueous solution with pH>7, the liposomes should be prepared in water (pH 5-6.5). The pH can be adjusted to 7.0-8.0 before linking to scFv-SH with 1 M HEPES buffer, pH 7.0-8.0, to facilitate the post-coating reaction.

3. Preparation of scFv-liposome-DNA Complexes

A. Pre-linking Method

scFv-SH was added to MPB-liposome at a protein/lipid (w/w) ratio of 1/5-1/40, preferably 1/10-1/20. The solution was mixed by gentle rotation for 30 min at room temperature to yield scFv-Lip. The scFv-Lip was used without purification although it can be purified by Sepharose CL-4B column chromatography. Plasmid DNA was diluted in water and added to the scFv-Lip at a DNA/lipid (μg/nmol) ratio of 1/6-1/20, preferably 1/10-1/14. The solution was mixed well for 5-15 min by inversion several times to produce scFv-Lip-DNA complex. scFv-Lip-DNA was used without purification although it can be purified by Sepharose CL-4B column chromatography. 80-100% of the scFv was found to be conjugated to the liposome.

B. Post-linking Method

Plasmid DNA was diluted in water and was added to the MPB-liposome at a DNA/lipid (μg/nmol) ratio of 1/6-1/20, preferably 1/10-1/14. The solution was mixed well for 5-15 min by inversion several times to produce an MPB-Lip-DNA complex. scFv-SH was then added to the complex at a protein/lipid (w/w) ratio of 1/5-1/40, preferably 1/10-1/20. The solution was mixed by gentle rotation for 30 min at room temperature, to produce the final scFv-Lip-DNA complex. The scFv-Lip-DNA was used without purification although it can be purified by Sepharose CL-4B column chromatography. 80-100% of the scFv was found to be conjugated to the liposome.

4. For intravenous injection, a 50% dextrose solution was added to the scFv-Lip-DNA to a final concentration of 5%.

Example 10

Immunoreactivity of Cysteine Containing TfRscFv-Immunoliposomes by the ELISA Assay

This example provides the characterization of the anti-TfRscFv-immunoliposomes produced by the conjugation method of this invention with respect to their ability to bind to TfR(+) cells *in vitro*. Human squamous cell carcinoma of head and neck cell line JSQ-3 served as the TfR(+) target cells for these studies.

As previously described in Example 4, indirect cellular enzyme-linked immunosorbent assay (ELISA) was employed to determine the immunoreactivity of the TfRscFv before and after conjugation to liposomes. Confluent JSQ-3 cells in 96-well plates were fixed with 0.5% glutaraldehyde in PBS for 10 min at room temperature. The plate was blocked with 5% fetal bovine serum (FBS) in PBS at 30°C for 30 min. The cysteine containing TfRscFv alone, this TfRscFv conjugated to cationic liposomes (TfRscFv-immunoliposomes) and untargeted liposomes were added to wells in triplicate. An anti-transferrin receptor monoclonal antibody (Hb21, obtained from David Fitzgerald, NIH) was used in one series of wells as a positive control. The plate was incubated at 4°C overnight. The wells were washed three times with PBS, and an anti-His monoclonal antibody (Qiagen) was added to each well (except for those receiving the antibody positive control) in 3% FBS in PBS and incubated at 37°C for 60 min. After three PBS washes, HRP-labeled goat-anti-mouse IgG (Sigma) diluted in 3% FBS was added to each well and incubated for 30 min at 37°C. The plate was washed three times with PBS and 100 µl substrate 0.4 mg/ml OPD in citrate phosphate buffer (Sigma) was added to each well. The color-development was stopped by adding 100 µl 2 M sulfuric acid to each well. The plate was read on an ELISA plate reader (Molecular Devices Corp.) at 490 nm.

Indirect cellular ELISA clearly demonstrated that the anti-TfR scFv containing a C-terminal cysteine maintained its immunoreactivity. The OD_{490} values increased with increasing amounts of TfRscFv protein, rising from 0.060 ± 0.0035 with 0.6 µg of protein, to 0.100 ± 0.0038 at 1.5 µg and 0.132 ± 0.0031 with 3 µg of TfRscFv. Moreover, this TfRscFv protein appears to have even greater binding activity than the parental Hb21 anti-transferrin receptor antibody used as a positive control. The OD_{490} for the highest concentration of the Hb21 (100 µl) was approximately 2-4 fold less (0.033 ± 0.0086).

The indirect cellular ELISA assay was also performed after the same TfRscFv protein was incorporated via the conjugation method of the invention (Example 9) into two different liposome complexes (Lip(A) and Lip(B)) to demonstrate the universality of this method with cationic liposomes. Both the pre- and post-linking conjugation methods of liposome preparation detailed in Example 9 were used. As shown in Table 8, the immunoreactivity of the TfRscFv prepared by the conjugation method is not lost through complexing to either of the two liposome compositions. This was true for both pre- and post-linking methods used to produce the immunoliposome complex. The TfRscFv-targeted lipoplexes also demonstrated binding to the

cells. This binding was significantly higher than that of the liposome without the TfRscFv, suggesting that this binding is in fact mediated through the attachment of the TfRscFv to the transferrin receptor on the cells.

Table 8

Binding of TfRscFv-immunoliposomes Prepared by the Conjugation Method to JSQ-3 Cells *In Vitro**

	DNA:Lipid Ratio	OD_{490}
Lip(B)-DNA	1:10	0.088
TfRscFv-Lip(A)-DNA by Pre-	1:10	0.152±0.016
TfRscFv-Lip(A)-DNA by Pre-	1:12	0.166±0.009
TfRscFv-Lip(A)-DNA by Post-	1:12	0.168±0.006
TfRscFv-Lip(B)-DNA by Pre-	1:12	0.139±0.012
TfRscFv only	--	0.235

* ELISA, OD_{490}, Mean ± SD (triplicate readings except for Lip(B)-DNA)
Pre- = Pre-linking Conjugation Method
Post- = Post-linking Conjugation Method

Example 11

Conjugated TfRscFv-immunoliposome Mediated Gene Transfection of Target Cells *In Vitro*

We determined the *in vitro* transfection efficiency of the TfRscFv-liposome complex, prepared by the conjugation method, in cells using the plasmid pLuc, which contains the firefly luciferase gene under control of the CMV promoter as the reporter gene. To demonstrate the universality of the TfRscFv as a targeting ligand, here also, as in Example 10, two separate liposome compositions (Lip(A) and Lip(B)) were conjugated to the TfRscFv protein. Human breast cancer cell line MDA-MB-435 and human squamous cell carcinoma of the head and neck cell line JSQ-3 were used in these studies. The *in vitro* transfection was performed in 24-well plates (Xu L. et al., *Hum. Gene Ther.* (1999) **10**:2941-2952). The transfection solutions were added to the cells in the presence of 10% serum. 24 hr later the cells were washed and lysed to measure the luciferase activity and protein concentration. The results are expressed as 10^3 relative light units (RLU) per μg protein in the lysate, as shown in Tables 9A and 9B.

Table 9A

Conjugated TfRscFv-immunoliposome Mediated Transfection *In Vitro*#

	Luciferase Activity (x 10^3 RLU/μg protein)	
	MDA-MB-435	JSQ-3
LipA	106	377
Tf-LipA	284	640
scFv-LipA*	560	1160
scFv-LipA**	660	1210
scFv-LipA (1/10)@	--	1315
scFv-LipA (1/20)@	--	751

Mean of duplicates
* Containing 5% MPB-DOPE
** Containing 7% MPB-DOPE
@ Ratio of scFv/lipids (w/w)

Table 9B

In Vitro Transfection Activity of Conjugated TfRscFv-Immunoliposome-DNA Complexes Prepared for Systemic Administration

	Luciferase Activity (x10^3 RLU/μg protein)	
	MDA-MB-435	JSQ-3
scFv-LipA-pLuc (pre-linking)*	58.4	675
scFv-LipA-pLuc (pre-linking)**	45.6	513
scFv-LipB-pLuc (pre-linking)*	51.4	415
scFv-LipA-pLuc (post-linking)*	58.1	856
scFv-LipA-pLuc (post-linking)**	45.3	343
scFv-LipB-pLuc (post-linking)*	47.2	237

* Containing 5% MPB-DOPE

** Containing 7% MPB-DOPE

The results show that the cysteine containing TfRscFv-immunoliposomes prepared by the conjugation method have very high transfection activity in vitro, 3-6 fold higher than the untargeted liposomes and 2-3 fold higher than the transferrin-targeted liposomes. This was true

for both liposome compositions and both human tumor cell lines. Thus, they still retain their immunoreactivity and can bind to their target receptor. Based upon Table 9A, the scFv-liposomes can also be used as efficient gene transfection reagents *in vitro*, and are much more efficient than commercially available cationic liposomes (DOTAP/DOPE and DDAB/DOPE) and transferrin-liposomes. The TfRscFv-immunoliposomes disclosed in the present invention can be used for an efficient *in vitro* gene transfection kit useful for the transfection of mammalian cells with transferrin receptors.

The TfRscFv is a smaller molecule than transferrin itself. Thus, the resulting complex is more compact and more easily taken up by the cells giving a higher transfection efficiency. These results are also advantageous for the use of the TfRscFv immunoliposome for systemic delivery for human use. The smaller size allows increased access to the tumor cells through the small capillaries. Most significantly, the TfRscFv is not a human blood product as is the Tf molecule. Therefore, the concerns and technical problems associated with the use of transferrin itself for human therapy are avoided.

Example 12

Conjugated TfRscFv-immunoliposome Mediated Expression of Wild-type p53 in a Nude Mouse Xenograft Model Following Systemic Delivery

In this example the ability of the TfRscFv, produced by the conjugation method of this invention, to direct a lipoplex carrying the wild-type p53 (wtp53) gene preferentially to tumor cells *in vivo* after systemic delivery is demonstrated. To demonstrate the universality of the TfRscFv as a targeting ligand, here also, as in Example 10, two separate liposome compositions (Lip(A) and Lip(B)) were complexed to the cysteine-containing TfRscFv protein by the conjugation method. Only the pre-linking method of conjugation as detailed in Example 9 was used in this study. 2.5×10^6 MDA-MB-435 human breast cancer cells were subcutaneously injected into 4-6 wk old female athymic nude mice. 1.1×10^7 DU145 human prostate cancer cells suspended in Matrigel® collagen basement membrane (Collaborative Biomedical Products) were also subcutaneously injected into 4-6 week old female athymic nude mice and tumors were allowed to develop. Animals bearing tumors of between 50-200 mm^3 were used in the study (1 animal/sample tested). Conjugated TfRscFv immunoliposomes carrying the wtp53 gene, as well as untargeted Lip(B)-p53 and wtp53 naked DNA were intravenously injected into the tail vein

of the animals. As an additional control, conjugated TfRscFv-Lip(A) carrying the empty vector in place of the p53 containing vector was also injected into a mouse. As described in Example 7, approximately 60 hours post-injection, the animals were sacrificed and the tumors, as well as the liver, were excised. Protein was isolated from the tissues and 100 μg of each sample (as determined by protein concentration assay) was run on a 10% polyacrylamide gel for Western blot analysis using an anti-p53 monoclonal antibody. In both of these tumor types the endogenous mouse and the exogenous human p53 migrate at the same position. The results here mirror those described in Example 7. As shown in Figure 9, both the DU145 and MDA-MB-435 tumors from the animals intravenously injected with the TfRscFv-Lip(A)-pCMVp53 lipoplex or the TfRscFv-Lip(B)-pCMVp53 lipoplex prepared by the conjugation method displayed a high level of expression of exogenous wtp53, as indicated by the intense p53 signal and an additional lower band, with the best expression in the DU145 tumors. While it appears that in both tumor types the Lip(A) composition was somewhat better than the Lip(B), both liposome compositions worked demonstrating the universality of this method. Only the endogenous mouse p53 protein was evident in the liver of these animals. In contrast, only the endogenous mouse p53 protein was evident in the tumors excised from the mice injected with the conjugated TfRscFv-Lip(B) carrying the empty vector or the naked wtp53 DNA. A small increase in p53 expression also was observed in the DU145 tumor with the untargeted Lip(B)-p53. Thus, the conjugated TfRscFv-immunoliposomes delivered the wtp53 gene preferentially to the tumors, as desired. It is also significant that this tumor targeting was evident in two different tumor types, indicating the general usefulness of the method of this invention. Therefore, the methods of this invention described in the preceding Examples generate a TfRscFv protein that not only retains its ability to bind to cationic liposomes but is still immunologically active preserving its ability to bind to the transferrin receptor *in vitro* and *in vivo*, thus fulfilling our objective of producing a tumor-specific, targeted immunoliposome for gene therapy.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An immunoliposome comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid.

2. The immunoliposome of claim 1 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

3. The immunoliposome of claim 1 wherein said nucleic acid is DNA.

4. The immunoliposome of claim 1 wherein said nucleic acid encodes a wild type p53.

5. The immunoliposome of claim 1 wherein said antibody or antibody fragment comprises a lipid tag.

6. The immunoliposome of claim 1 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a sulfhydryl group at a carboxy terminus on said antibody or antibody fragment.

7. The immunoliposome of claim 6 wherein said sulfur atom is part of a cysteine residue.

8. The immunoliposome of claim 6 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

9. The immunoliposome of claim 1 wherein said antibody fragment is a single chain.

10. The immunoliposome of claim 1 wherein said antibody or antibody fragment and said cationic liposome are present at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

11. The immunoliposome of claim 1 wherein said nucleic acid and said cationic liposome are present at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

12. A pharmaceutical composition comprising the immunoliposome of claim 1.

13. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) mixing nucleic acid encoding a wild type p53 with a cationic liposome to produce a nucleic acid-liposome complex;

b) preparing an antibody or antibody fragment capable of binding to a transferrin receptor; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex.

14. The method of claim 13 wherein said antibody or antibody fragment comprises a lipid tag.

15. The method of claim 13 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

16. The method of claim 15 wherein said reducible group is a sulfhydryl.

17. The method of claim 16 wherein said sulfhydryl is part of a cysteine residue.

18. The method of claim 15 wherein said cationic liposome comprises DOPE linked to MPB or other sulfhydryl reacting group.

19. The method of claim 13 wherein said nucleic acid is DNA.

20. The method of claim 13 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

21. The method of claim 13 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

22. The method of claim 13 wherein said antibody fragment is a single chain.

23. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) preparing an antibody or antibody fragment capable of binding to a transferrin receptor;

b) mixing said antibody or antibody fragment with a cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with nucleic acid encoding a wild type p53 to form said nucleic acid-cationic immunoliposome complex.

24. The method of claim 23 wherein said antibody or antibody fragment comprises a lipid tag.

25. The method of claim 23 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

26. The method of claim 25 wherein said reducible group is a sulfhydryl.

27. The method of claim 26 wherein said sulfhydryl is part of a cysteine residue.

28 The method of claim 25 wherein said cationic liposome comprises MPB-DOPE.

29. The method of claim 23 wherein said nucleic acid is DNA.

30. The method of claim 23 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

31. The method of claim 23 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

32. The method of claim 23 wherein said antibody fragment is a single chain.

33. A method for providing a therapeutic molecule to an animal in need thereof, comprising administering to said animal a therapeutically effective amount of a nucleic acid-cationic immunoliposome complex comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid.

34. The method of claim 33 wherein said complex is administered systemically.

35. The method of claim 33 wherein said complex is administered intravenously.

36. The method of claim 33 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

37. The method of claim 33 wherein said antibody fragment is a single chain.

38. The method of claim 33 wherein said nucleic acid is DNA.

39. The method of claim 33 wherein said nucleic acid encodes a wild type p53.

40. The method of claim 33 wherein said antibody or antibody fragment comprises a lipid tag.

41. The method of claim 33 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a reducible group at a carboxy terminus on said antibody or antibody fragment.

42. The method of claim 41 wherein said reducible group is a sulfhydryl.

43. The method of claim 42 wherein said sulfhydryl is part of a cysteine residue.

44. The method of claim 41 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

45. The method according to claim 33 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

46. The method according to claim 33 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

47. The method according to claim 33 wherein said animal is a human.

48. The method according to claim 33 wherein said animal has cancer.

49. The method according to claim 48 wherein said cancer is selected from the group consisting of i) head and neck cancer, ii) breast cancer and iii) prostate cancer.

50. A kit comprising a cationic immunoliposome wherein said cationic immunoliposome comprises a transferrin receptor binding antibody fragment.

51. The kit of claim 50 wherein said antibody fragment is a single chain.

52. The kit of claim 50 wherein said antibody fragment comprises a lipid tag.

53. The kit of claim 50 wherein said antibody fragment is conjugated to a cationic liposome.

54. The kit of claim 50 said antibody fragment and cationic lipids are present in a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

55. The kit of claim 50 wherein said cationic immunoliposome is in an aqueous solution.

56. The kit of claim 50 further comprising a nucleic acid for use as a positive control in a container separate from said cationic immunoliposome.

57. The kit of claim 56 wherein said nucleic acid encodes a reporter gene selected from the group consisting of luciferase, β-galactosidase and green fluorescent protein.

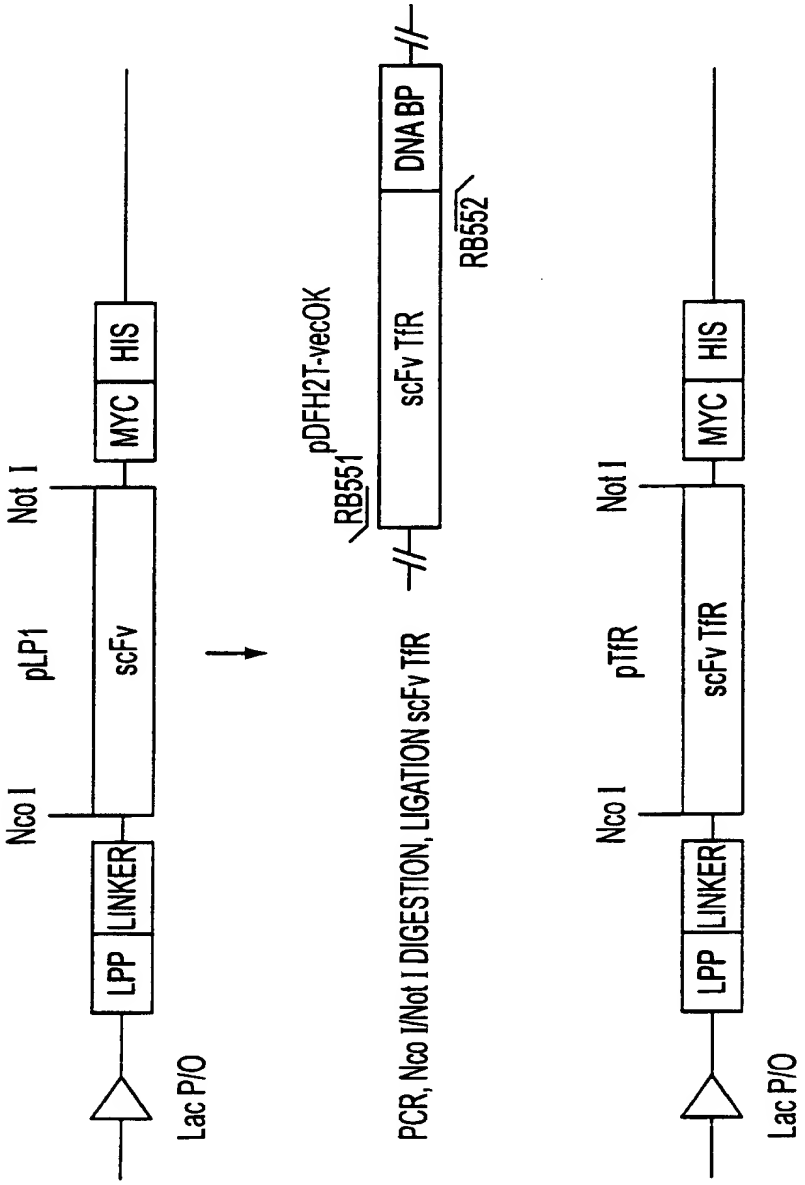
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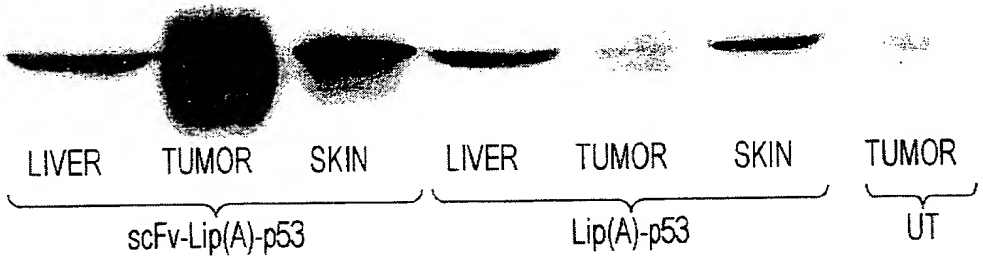
FIG. 1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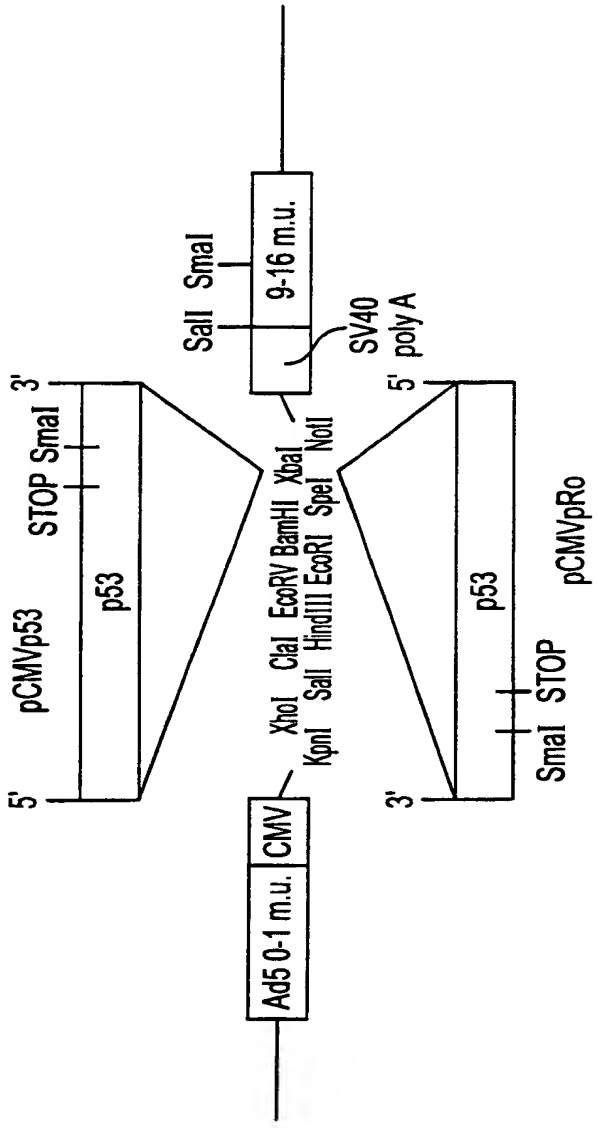
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p53
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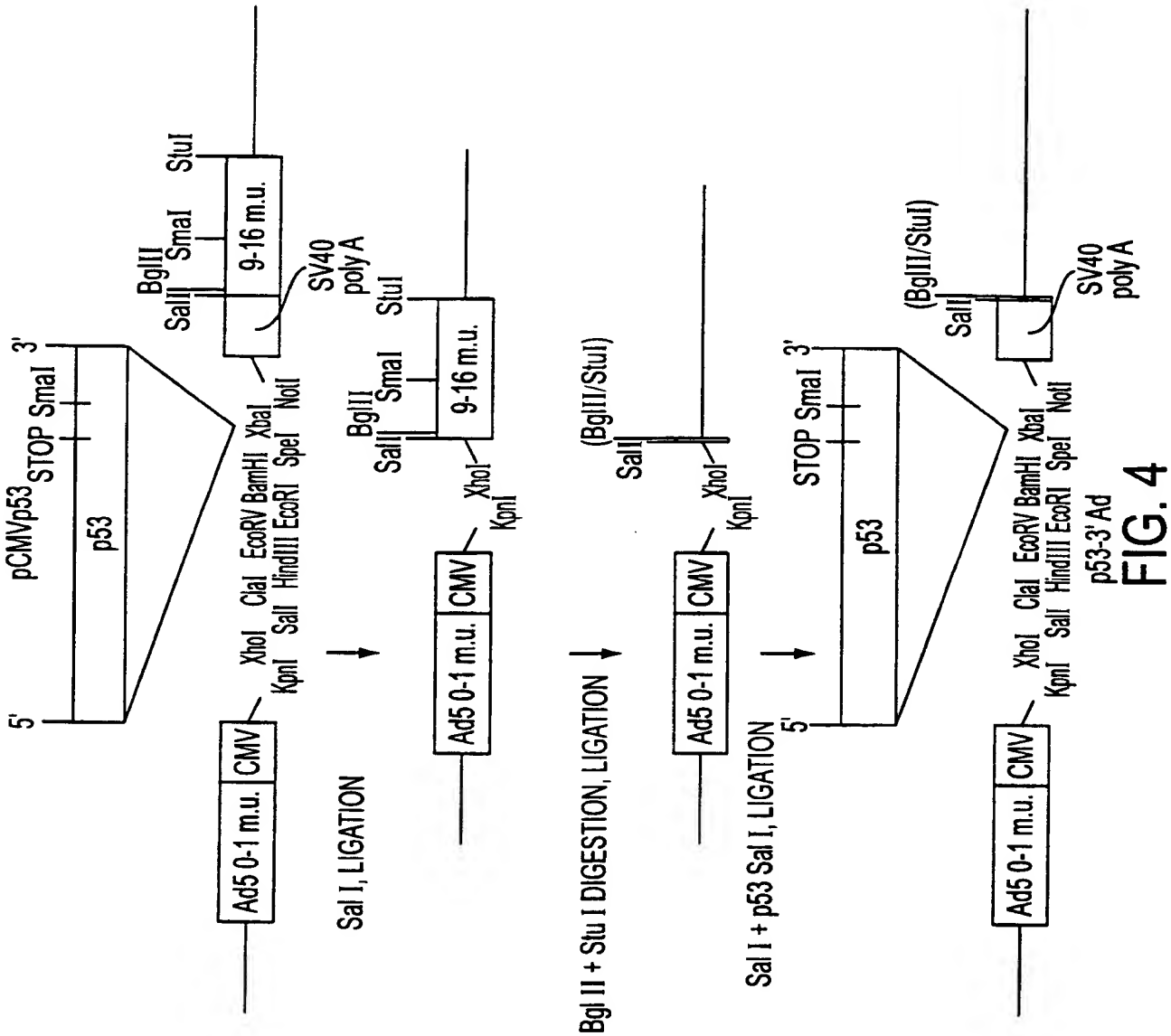
FIG. 3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BglII SalI SmaI StuI
9-16 m.u.
SV40 poly A
Sal I, LIGATION
Bgl II + Stu I DIGESTION, LIGATION
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FIG. 4

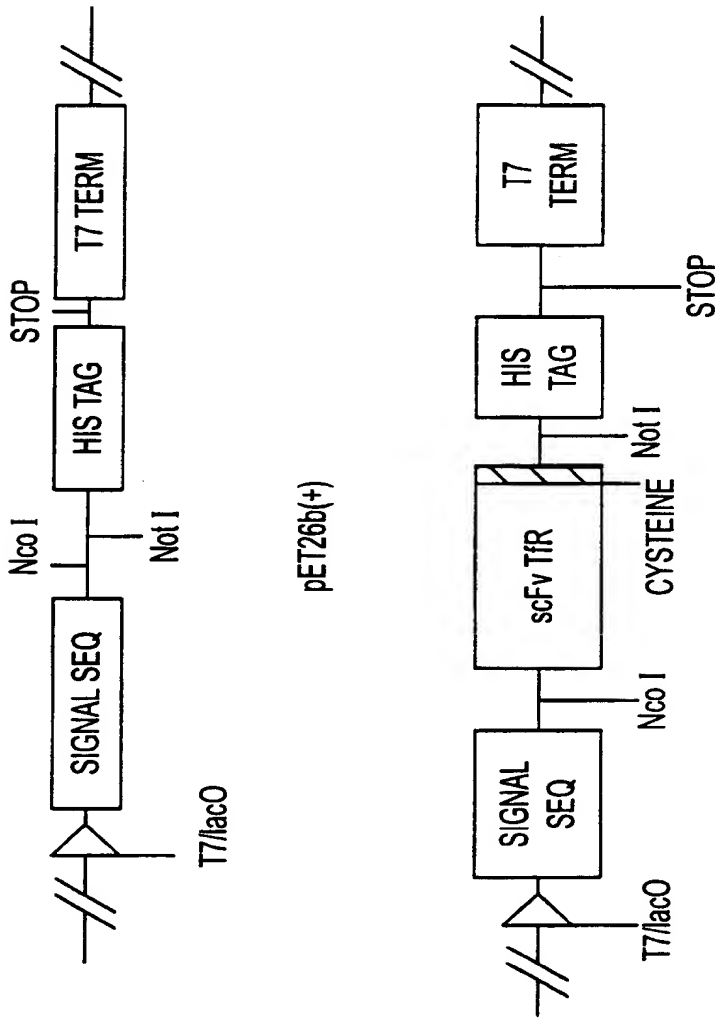
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FIG. 5

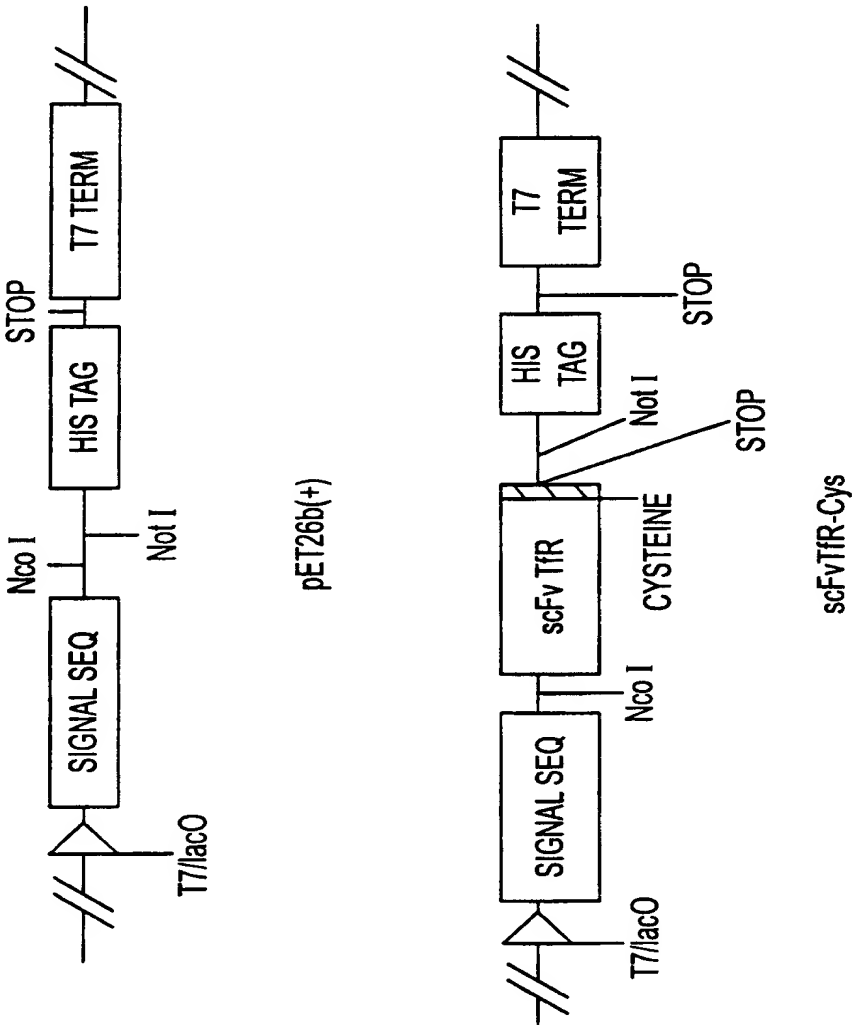
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FIG. 6

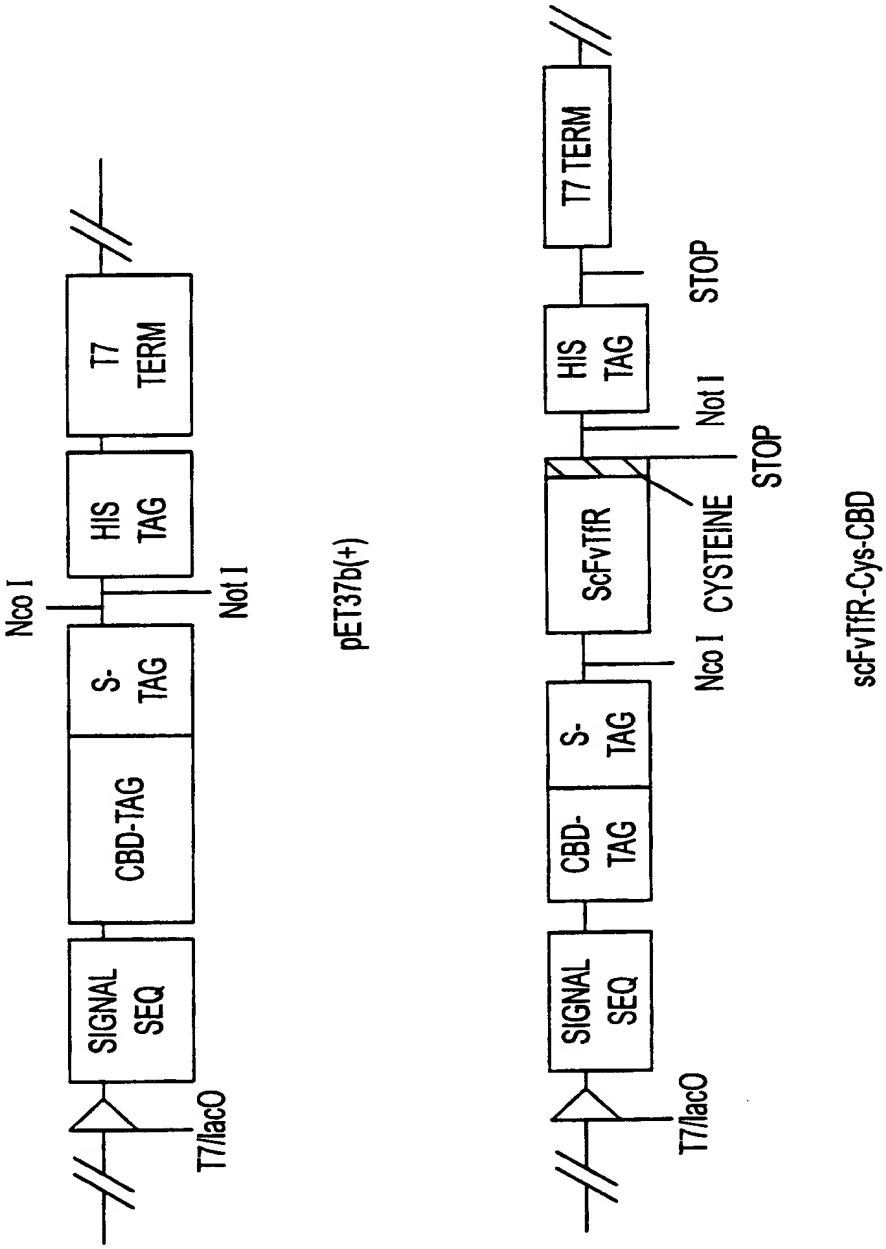
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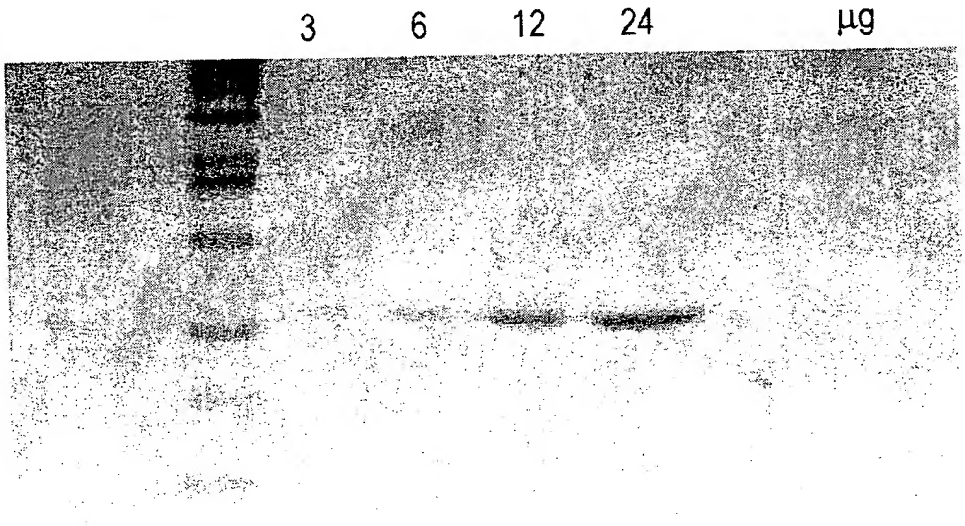
FIG. 7



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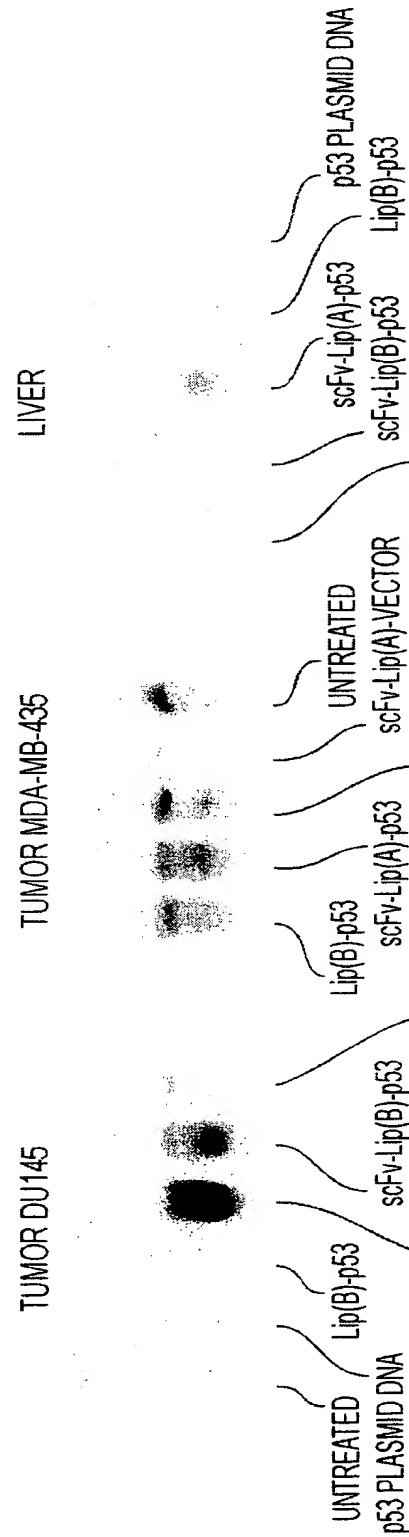
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TUMOR MDA-MB-435
LIVER
UNTREATED
p53 PLASMID DNA
Lip(B)-p53
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scFv-Lip(A)-VECTOR
Lip(B)-p53
scFv-Lip(A)-p53
scFv-Lip(B)-p53
scFv-Lip(A)-VECTOR
UNTREATED
scFv-Lip(A)-VECTOR
scFv-Lip(B)-p53
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Lip(B)-p53
p53 PLASMID DNA

FIG. 9

SEQUENCE LISTING

<110> Xu, Liang
Huang, Cheng-Cheng
Alexander, William
Tang, WenHua
Chang, Esther H.
Georgetown University
SynerGene Therapeutics, Inc.

<120> ANTIBODY FRAGMENT-TARGETED IMMUNOLIPOSOMES FOR SYSTEMIC GENE DELIVERY

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04392

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 C12N15/88 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COMPAGNON B ET AL: "ENHANCED GENE DELIVERY AND EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS BY CATIONIC IMMUNOLIPOSOMES" JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 7, no. 1, 1997, pages 127-141, XP000682912 ISSN: 0898-2104 the whole document --- -/--	1-57

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2000	14/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niemann, F

3

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA J ET AL: "SIMPLE PREPARATION AND CHARACTERIZATION OF CATIONIC LIPOSOMES ASSOCIATED WITH A MONOCLONAL ANTIBODY AGAINST GLIOMA-ASSOCIATED ANTIGEN (IMMUNOLIPOSOMES)" JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 5, no. 4, 1995, pages 981-995, XP000558625 ISSN: 0898-2104 the whole document	1-57
A	XU L ET AL: "Transferrin-liposome-mediated p53 sensitization of squamous cell carcinoma of the head and neck to radiation in vitro" HUMAN GENE THERAPY,XX,XX, vol. 8, no. 4, 1 March 1997 (1997-03-01), pages 467-475, XP002098089 ISSN: 1043-0342 cited in the application the whole document	4,13,23, 39,48,49
A	JIANG AN ET AL: "Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies." JOURNAL OF VIROLOGY, vol. 72, no. 12, December 1998 (1998-12), pages 10148-10156, XP002103152 ISSN: 0022-538X cited in the application the whole document	2,9,13, 22,23, 32,36, 37,50,51
A	J DE KRUIF ET AL: "Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes" FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 3, no. 399, 16 December 1996 (1996-12-16), pages 232-236, XP002075140 ISSN: 0014-5793 the whole document	5,9,14, 22,24, 32,37, 40,51,52

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04392

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	KOBATAKE E ET AL: "A FLUOROIMMUNOASSAY BASED ON IMMUNOLIPOSOMES CONTAINING GENETICALLYENGINEERED LIPID-TAGGED ANTIBODY" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 7, 1 April 1997 (1997-04-01), pages 1295-1298, XP000689729 ISSN: 0003-2700 abstract	5,9,14, 22,24, 32,37, 40,51,52
A	WO 83 02069 A (UNIV CALIFORNIA) 23 June 1983 (1983-06-23) the whole document	6-8, 15-18, 25-28, 41-44,53

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/04392

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8302069 A	23-06-1983	US 4429008 A	31-01-1984
		CA 1197190 A	26-11-1985
		EP 0097691 A	11-01-1984

09191405

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2444-105.PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/04392	22/02/2000	22/02/1999
International Patent Classification (IPC) or national classification and IPC A61K9/127		
Applicant GEORGETOWN UNIVERSITY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 11 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/09/2000	29.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Pregetter, M Telephone No. +49 89 2399 8719

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/04392

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-26 as originally filed

Claims, No.:

1-68 with telefax of 02/03/2001

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
see separate sheet

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 33-49, concerning industrial applicability.

because:

☒ the said international application, or the said claims Nos. 33-49 relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N) Yes: Claims 2,4,5,9,13-49,51,52,56,57

	No:	Claims	1,3,6-8,10-12,50,53-55
Inventive step (IS)	Yes:	Claims	13-32,56,57
	No:	Claims	1-12,33-55
Industrial applicability (IA)	Yes:	Claims	1-32,50-57
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I
Basis of the report

The amendments filed with the letter dated 02.03.2001 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

The most general disclosure of a method of preparing immunoliposomes is defined in claims 13 and 23. The description on p.4, l.19-p.7, l.19 discloses only methods using scFv liposomes complexed with DNA under very specific conditions. The rest of the description describes specific examples.
A general disclosure for a method for preparing cationic immunoliposomes with any antibodies/antibody fragments and any nucleic acid cannot be found in the application as filed.

This objection concerns all the independent claims.

The international preliminary examination report has been established on the application as originally filed.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 33-49 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V
Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

D1: COMPAGNON B ET AL: 'ENHANCED GENE DELIVERY AND EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS BY CATIONIC IMMUNOLIPOSOMES' JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 7, no. 1, 1997, pages 127-141, XP000682912 ISSN: 0898-2104

D2: YOSHIDA J ET AL: 'SIMPLE PREPARATION AND CHARACTERIZATION OF CATIONIC LIPOSOMES ASSOCIATED WITH A MONOCLONAL ANTIBODY AGAINST GLIOMA-ASSOCIATED ANTIGEN (IMMUNOLIPOSOMES)' JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 5, no. 4, 1995, pages 981-995, XP000558625 ISSN: 0898-2104

D3: XU L ET AL: 'Transferrin-liposome-mediated p53 sensitization of squamous cell carcinoma of the head and neck to radiation in vitro' HUMAN GENE THERAPY,XX,XX, vol. 8, no. 4, 1 March 1997 (1997-03-01), pages 467-475, XP002098089 ISSN: 1043-0342 cited in the application

D4: JIANG AN ET AL: 'Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies.' JOURNAL OF VIROLOGY, vol. 72, no. 12, December 1998 (1998-12), pages 10148-10156, XP002103152 ISSN: 0022-538X cited in the application

D5: J DE KRUIF ET AL: 'Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes' FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 3, no. 399, 16 December 1996 (1996-12-16), pages 232-236, XP002075140 ISSN: 0014-5793

D6: KOBATAKE E ET AL: 'A FLUOROIMMUNOASSAY BASED ON IMMUNOLIPOSOMES CONTAINING GENETICALLYENGINEERED LIPID-TAGGED ANTIBODY' ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 7, 1 April 1997 (1997-04-01), pages 1295-1298, XP000689729 ISSN: 0003-2700

D7: WO 83 02069 A (UNIV CALIFORNIA) 23 June 1983 (1983-06-23)

2. The subject-matter of present claim 1 is not novel according to Article 33(2) PCT.

2.1. Immunoliposomes comprising cationic liposomes, antibodies or antibody fragments and nuclei acids are already known from documents D1 and D2.

D1 (cf. materials and methods) discloses cationic liposomes comprising DOTAB, eggPC and DOPE. As link for the covalent bonding of an antibody or antibody fragment the liposomes comprise MMCC-DHPE, a maleimido-derivative. The covalently bonded antibody AF-20 recognizes a glycoprotein which is expressed on the surface of human hepatocellular carcinoma cells or other cancer cells. The nuclei acid is DNA.
D2 (cf. materials and methods) also describes cationic immunoliposomes. The liposomes consist of TMAG, DLPC and DOPE. Plasmid DNA is associated with these liposomes. A G-22 monoclonal antibody is used to target the immunoliposomes to human glioma cells.

2.2. The dependent claims 2-11 do not contain any features which, in combination with the features of any claim to which they refer, might establish novelty and an inventive step over D1-D7 (Articles 33(2) and 33(3) PCT). These claims are only allowable in combination with patentable independent claims.
ad claim 2: D4 suggests the use of antibodies against the transferrin receptor (TFR). There cannot be seen an inventive step in replacing one antibody known for use in gene transfer by another antibody well known for the same purpose in a slightly different transfer system.
ad claim 3: cf. 2.1.
ad claim 4: It is well known to transfer wt p53 to cancer cells. There cannot be seen an inventive step in transferring a gene well known to affect tumour progression.
ad claim 5: The problem of anchoring an antibody in a positively charged liposome via lipid tags has been already addressed in D5. Also, D6 describes the lipid-tagging of antibodies for anchoring in a lipid bilayer. Since the problem of the lipid-modifying of antibodies is not directly connected with the idea of providing immunoliposomes comprising cationic liposomes, antibodies and nucleic acids, no inventive step is present.
ad claims 6-8: cf. D1, p.129, third paragraph - p.130, first paragraph.
ad claim 9: Single chain antibody derivatives are well known in the art and have been used several times for the targeting of liposomes and even viruses (D5, D6, D7, D4).
ad claim 10: cf. D2, p.984, "Preparation of Immunoliposomes with the Entrapped Plasmids".

ad claim 11: cf. D1, fig 2.

3. The subject-matter of claim 12 is not new according to Article 33(2) PCT.
Both D1 and D2 describe the cationic immunoliposomes in a buffered solution which is well suited for the application as a pharmaceutical composition.

4.1. Document D2, which is considered to represent the most relevant state of the art, discloses a method for providing a therapeutic molecule from which the subject-matter of claim 33 differs in that the method is directed towards an animal.

4.2. The subject-matter of claim 33 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
Although document D2 does not disclose a method for providing a therapeutic molecule to an animal, it does disclose in vitro transfection and does suggest to use the nucleic-acid immunoliposomes (cf. 2.1) as a DNA delivery system for humans (p.989, last paragraph + p.992 last paragraph).
A person skilled in the art would, without any doubt, follow this suggestion and administer such nuclei-acid immunoliposomes to animals/humans.

4.3. The dependent claims 34-49 do not contain additional technical features which might establish an inventive step over D1 to D7 (article 33(3) PCT). These claims are only allowable in combination with patentable independent claims.
ad claims 34-35: These claims define only usual administration methods.
ad claims 36-49: cf. points 2.1 and 2.2.

5. The subject-matter of claim 50 is not new according to Article 33(2) PCT.
For the interpretation of claim 50 confer item VIII, point 1.

5.1. The disclosure of documents D1 and D2 has already been discussed under item V, point 2.1. The presence of a container is implicit.

5.2. Furthermore, cationic immunoliposomes are disclosed in document D5. D5 discloses cationic immunoliposomes comprising lipid modified single chain anti-IgG and anti-CD fragments in a protein:lipid ratio (w:w) of about 1:20 (p. 233, point 2.5.). The immunoliposomes are suspended in an aqueous HEPES buffer.

5.3. The dependent claims 51-55 do not contain any features which, in combination with the features of any claim to which they refer, might establish novelty and an

inventive step over D1-D7 (Articles 33(2) and 33(3) PCT). The disclosures of these documents has already been discussed under points 2.1, 2.2, 5.1 and 5.2. These claims are only allowable in combination with patentable independent claims.

6.1. Document D2, which is considered to represent the most relevant state of the art, discloses (cf. p.984, first paragraph) a method for the preparation of nucleic acid-immunoliposomes from which the subject-matter of claim 13 differs in that a different process in defined.

The subject-matter of claim 13 is therefore novel (Article 33(2) PCT).

6.2. The problem to be solved by the present invention may therefore be regarded as: How to provide a process for the preparation of nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

6.3. The solution to this problem proposed in claim 13 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
None of the documents cited in the search report suggests to reverse the preparation steps (i.e. incubation of liposome with nucleic acid prior to the attachment of the antibody/antibody fragment). Furthermore, the combination of wt p53 with an antibody/antibody fragment capable of binding to a transferrin receptor has not been suggested.

6.4. Claims 14-22 are dependent on claim 13 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

7.1. Document D1, which is considered to represent the most relevant state of the art, discloses (cf. p.130, last paragraph) a method for the preparation of nucleic acid-cationic immunoliposomes from which the subject-matter of claim 23 differs in that a different antibody/antibody fragment and a different nucleic acid is used.

The subject-matter of claim 23 is therefore novel (Article 33(2) PCT).

7.2. The problem to be solved by the present invention may therefore be regarded as: How to provide a process for the preparation of nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

7.3. The solution to this problem proposed in claim 23 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
Although the method of preparation has already been described in principal by D1, there is no suggestion in the documents cited by the search report to prepare nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.
Document D3 suggests the use of wt p53 in gene therapy in combination with transferrin. The use of an antibody fragment capable of binding to a transferrin receptor is suggested in D4 (p.10148, last paragraph) only in connection with retroviral vectors and not in connection with liposomes.
Therefore, there are no directions for a person skilled in the art to prepare nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

7.4. Claims 24-32 are dependent on claim 23 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

8. None of the documents cited in the search report discloses or even suggests a kit comprising two separate containers, one comprising a cationic immunoliposome, the other a nucleic acid as defined in claim 56.

9. For the assessment of the present claims 33-49 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

1. The subject-matter of present claim 50 is not clear (Article 6 PCT). A kit is generally defined by comprising at least two parts that can be considered to represent two distinct entities, e.g. a packaging unit and its contents or two different, physically separate, solutions.
   Present claim 50 defines only one of the at least two parts (a cationic immunoliposome). A second part has not been defined, and therefore does not present any restriction. At present, the second part is taken to be any container that can contain the cationic immunoliposomes.

2. The vague and imprecise statement in the description on page 26, last paragraph, implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

3. The description is not in accordance with the claims (Article 6 PCT).

WHAT IS CLAIMED IS:

1. A nucleic acid-cationic immunoliposome complex comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid wherein said nucleic acid-cationic immunoliposome complex is prepared by a method comprising the steps of:

1) a) mixing said nucleic acid with said cationic liposome to produce a nucleic acid-liposome complex;

b) preparing said antibody or antibody fragment; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex; or

2) a) preparing said antibody or antibody fragment;

b) mixing said antibody or antibody fragment with said cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with said nucleic acid to form said nucleic acid-cationic immunoliposome complex.

2. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

3. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said nucleic acid is DNA.

4. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said nucleic acid encodes a wild type p53.

5. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said antibody or antibody fragment comprises a lipid tag.

6. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a sulfhydryl group at a carboxy terminus on said antibody or antibody fragment.

7. The nucleic acid-cationic immunoliposome complex of claim 6 wherein said sulfur atom is part of a cysteine residue.

8. The nucleic acid-cationic immunoliposome complex of claim 6 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

9. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said antibody fragment is a single chain.

10. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said antibody or antibody fragment and said cationic liposome are present at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

11. The nucleic acid-cationic immunoliposome complex of claim 1 wherein said nucleic acid and said cationic liposome are present at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

12. A pharmaceutical composition comprising the nucleic acid-cationic immunoliposome complex of claim 1.

13. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) mixing nucleic acid with a cationic liposome to produce a nucleic acid-liposome complex;

b) preparing an antibody or antibody fragment; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex.

14. The method of claim 13 wherein said nucleic acid encodes a wild type p53.

15. The method of claim 13 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

16. The method of claim 13 wherein said antibody or antibody fragment comprises a lipid tag.

17. The method of claim 13 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

18. The method of claim 17 wherein said reducible group is a sulfhydryl.

19. The method of claim 18 wherein said sulfhydryl is part of a cysteine residue.

20. The method of claim 17 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom of said reducible group.

21. The method of claim 17 wherein said cationic liposome comprises DOPE linked to MPB or other sulfhydryl reacting group.

22. The method of claim 13 wherein said nucleic acid is DNA.

23. The method of claim 13 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

24. The method of claim 13 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

25. The method of claim 13 wherein said antibody fragment is a single chain.

26. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) preparing an antibody or antibody fragment;

b) mixing said antibody or antibody fragment with a cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with nucleic acid to form said nucleic acid-cationic immunoliposome complex.

27. The method of claim 26 wherein said nucleic acid encodes a wild type p53.

28. The method of claim 26 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

29. The method of claim 26 wherein said antibody or antibody fragment comprises a lipid tag.

30. The method of claim 26 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

31. The method of claim 30 wherein said reducible group is a sulfhydryl.

32. The method of claim 31 wherein said sulfhydryl is part of a cysteine residue.

33. The method of claim 31 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom of said reducible group.

34. The method of claim 30 wherein said cationic liposome comprises MPB-DOPE.

35. The method of claim 26 wherein said nucleic acid is DNA.

36. The method of claim 26 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

37. The method of claim 26 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

38. The method of claim 26 wherein said antibody fragment is a single chain.

39. A method for providing a therapeutic molecule to an animal in need thereof, comprising administering to said animal a therapeutically effective amount of a nucleic acid-cationic immunoliposome complex comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid wherein said nucleic acid-cationic immunoliposome complex is prepared by a method comprising the steps of:

1) a) mixing said nucleic acid with said cationic liposome to produce a nucleic acid-liposome complex;

b) preparing said antibody or antibody fragment; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex; or

2) a) preparing said antibody or antibody fragment;

b) mixing said antibody or antibody fragment with a cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with said nucleic acid to form said nucleic acid-cationic immunoliposome complex.

40. The method of claim 39 wherein said complex is administered systemically.

41. The method of claim 39 wherein said complex is administered intravenously.

42. The method of claim 39 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

43. The method of claim 39 wherein said antibody fragment is a single chain.

44. The method of claim 39 wherein said nucleic acid is DNA.

45. The method of claim 39 wherein said nucleic acid encodes a wild type p53.

46. The method of claim 39 wherein said antibody or antibody fragment comprises a lipid tag.

47. The method of claim 39 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a reducible group at a carboxy terminus on said antibody or antibody fragment.

48. The method of claim 47 wherein said reducible group is a sulfhydryl.

49. The method of claim 48 wherein said sulfhydryl is part of a cysteine residue.

50. The method of claim 47 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

51. The method according to claim 39 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

52. The method according to claim 39 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

53. The method according to claim 39 wherein said animal is a human.

54. The method according to claim 39 wherein said animal has cancer.

55. The method according to claim 54 wherein said cancer is selected from the group consisting of i) head and neck cancer, ii) breast cancer and iii) prostate cancer.

56. A kit comprising

i) a nucleic acid;

ii) a cationic immunoliposome; and

iii) an instruction manual for preparing a nucleic acid-cationic immunoliposome complex prepared by a method comprising the steps of:

1) a) mixing said nucleic acid with said cationic liposome to produce a nucleic acid-liposome complex;

b) preparing an antibody or antibody fragment; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex; or

2) a) preparing an antibody or antibody fragment;

b) mixing said antibody or antibody fragment with said cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with said nucleic acid to form said nucleic acid-cationic immunoliposome complex.

57. The kit of claim 56 wherein said nucleic acid encodes a wild type p53.

58. The kit of claim 56 wherein said cationic liposome comprises an antibody or antibody fragment capable of binding to a transferrin receptor.

59. The kit of claim 56 wherein said antibody fragment is a single chain.

60. The kit of claim 56 wherein said antibody fragment comprises a lipid tag.

61. The kit of claim 56 wherein said antibody fragment is conjugated to a cationic liposome.

62. The kit of claim 56 said antibody fragment and cationic lipids are present in a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

63. The kit of claim 56 wherein said cationic immunoliposome is in an aqueous solution.

64. The kit of claim 56 further comprising a nucleic acid for use as a positive control in a container separate from said cationic immunoliposome.

65. The kit of claim 64 wherein said nucleic acid encodes a reporter gene selected from the group consisting of luciferase, β-galactosidase and green fluorescent protein.

66. A method of transfecting cells with a desired nucleic acid wherein said method comprises administering the nucleic acid-cationic immunoliposome complex of the kit of claim 56 to said cells wherein said complex comprises said desired nucleic acid.

67. The method of claim 66 wherein said method is performed in vitro.

68. A method of transfecting cells in a tissue in an animal with a desired nucleic acid wherein said method comprises administering the nucleic acid-cationic immunoliposome complex of the kit of claim 56 to said cells wherein said complex comprises said desired nucleic acid.

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

SAXE, Stephen A. et al.
ROTHWELL, FIGG, ERNST
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NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT

Date of mailing *(day/month/year)* 29.05.2001

Applicant's or agent's file reference
2444-105.PCT

IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/04392	22/02/2000	22/02/1999

Applicant
GEORGETOWN UNIVERSITY et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
2444-105.PCT		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US00/04392	22/02/2000	22/02/1999
International Patent Classification (IPC) or national classification and IPC A61K9/127		
Applicant GEORGETOWN UNIVERSITY et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 11 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 8 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/09/2000	29.05.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Pregetter, M Telephone No. +49 89 2399 8719

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/US00/04392

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-26 as originally filed

Claims, No.:

1-68 with telefax of 02/03/2001

Drawings, sheets:

1/9-9/9 as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☒ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)
   see separate sheet

6. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 33-49, concerning industrial applicability.

because:

   ☒ the said international application, or the said claims Nos. 33-49 relate to the following subject matter which does not require an international preliminary examination (*specify*):
   see separate sheet

   ☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

   ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

   ☐ no international search report has been established for the said claims Nos. .

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

   ☐ the written form has not been furnished or does not comply with the standard.

   ☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

   Novelty (N) Yes: Claims 2,4,5,9,13-49,51,52,56,57

	No:	Claims	1,3,6-8,10-12,50,53-55
Inventive step (IS)	Yes:	Claims	13-32,56,57
	No:	Claims	1-12,33-55
Industrial applicability (IA)	Yes:	Claims	1-32,50-57
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item I

Basis of the report

The amendments filed with the letter dated 02.03.2001 introduce subject-matter which extends beyond the content of the application as filed, contrary to Article 34(2)(b) PCT. The amendments concerned are the following:

The most general disclosure of a method of preparing immunoliposomes is defined in claims 13 and 23. The description on p.4, l.19-p.7, l.19 discloses only methods using scFv liposomes complexed with DNA under very specific conditions. The rest of the description describes specific examples.
A general disclosure for a method for preparing cationic immunoliposomes with any antibodies/antibody fragments and any nucleic acid cannot be found in the application as filed.

This objection concerns all the independent claims.

The international preliminary examination report has been established on the application as originally filed.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

Claims 33-49 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

D1: COMPAGNON B ET AL: 'ENHANCED GENE DELIVERY AND EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS BY CATIONIC IMMUNOLIPOSOMES' JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 7, no. 1, 1997, pages 127-141, XP000682912 ISSN: 0898-2104

D2: YOSHIDA J ET AL: 'SIMPLE PREPARATION AND CHARACTERIZATION OF CATIONIC LIPOSOMES ASSOCIATED WITH A MONOCLONAL ANTIBODY AGAINST GLIOMA-ASSOCIATED ANTIGEN (IMMUNOLIPOSOMES)' JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 5, no. 4, 1995, pages 981-995, XP000558625 ISSN: 0898-2104

D3: XU L ET AL: 'Transferrin-liposome-mediated p53 sensitization of squamous cell carcinoma of the head and neck to radiation in vitro' HUMAN GENE THERAPY,XX,XX, vol. 8, no. 4, 1 March 1997 (1997-03-01), pages 467-475, XP002098089 ISSN: 1043-0342 cited in the application

D4: JIANG AN ET AL: 'Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies.' JOURNAL OF VIROLOGY, vol. 72, no. 12, December 1998 (1998-12), pages 10148-10156, XP002103152 ISSN: 0022-538X cited in the application

D5: J DE KRUIF ET AL: 'Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes' FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 3, no. 399, 16 December 1996 (1996-12-16), pages 232-236, XP002075140 ISSN: 0014-5793

D6: KOBATAKE E ET AL: 'A FLUOROIMMUNOASSAY BASED ON IMMUNOLIPOSOMES CONTAINING GENETICALLYENGINEERED LIPID-TAGGED ANTIBODY' ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 7, 1 April 1997 (1997-04-01), pages 1295-1298, XP000689729 ISSN: 0003-2700

D7: WO 83 02069 A (UNIV CALIFORNIA) 23 June 1983 (1983-06-23)

2. The subject-matter of present claim 1 is not novel according to Article 33(2) PCT.

2.1. Immunoliposomes comprising cationic liposomes, antibodies or antibody fragments and nuclei acids are already known from documents D1 and D2.

D1 (cf. materials and methods) discloses cationic liposomes comprising DOTAB, eggPC and DOPE. As link for the covalent bonding of an antibody or antibody fragment the liposomes comprise MMCC-DHPE, a maleimido-derivative. The covalently bonded antibody AF-20 recognizes a glycoprotein which is expressed on the surface of human hepatocellular carcinoma cells or other cancer cells. The nuclei acid is DNA.
D2 (cf. materials and methods) also describes cationic immunoliposomes. The liposomes consist of TMAG, DLPC and DOPE. Plasmid DNA is associated with these liposomes. A G-22 monoclonal antibody is used to target the immunoliposomes to human glioma cells.

2.2. The dependent claims 2-11 do not contain any features which, in combination with the features of any claim to which they refer, might establish novelty and an inventive step over D1-D7 (Articles 33(2) and 33(3) PCT). These claims are only allowable in combination with patentable independent claims.
ad claim 2: D4 suggests the use of antibodies against the transferrin receptor (TFR). There cannot be seen an inventive step in replacing one antibody known for use in gene transfer by another antibody well known for the same purpose in a slightly different transfer system.
ad claim 3: cf. 2.1.
ad claim 4: It is well known to transfer wt p53 to cancer cells. There cannot be seen an inventive step in transferring a gene well known to affect tumour progression.
ad claim 5: The problem of anchoring an antibody in a positively charged liposome via lipid tags has been already addressed in D5. Also, D6 describes the lipid-tagging of antibodies for anchoring in a lipid bilayer. Since the problem of the lipid-modifying of antibodies is not directly connected with the idea of providing immunoliposomes comprising cationic liposomes, antibodies and nucleic acids, no inventive step is present.
ad claims 6-8: cf. D1, p.129, third paragraph - p.130, first paragraph.
ad claim 9: Single chain antibody derivatives are well known in the art and have been used several times for the targeting of liposomes and even viruses (D5, D6, D7, D4).
ad claim 10: cf. D2, p.984, "Preparation of Immunoliposomes with the Entrapped Plasmids".

ad claim 11: cf. D1, fig 2.

3. The subject-matter of claim 12 is not new according to Article 33(2) PCT.
Both D1 and D2 describe the cationic immunoliposomes in a buffered solution which is well suited for the application as a pharmaceutical composition.

4.1. Document D2, which is considered to represent the most relevant state of the art, discloses a method for providing a therapeutic molecule from which the subject-matter of claim 33 differs in that the method is directed towards an animal.

4.2. The subject-matter of claim 33 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
Although document D2 does not disclose a method for providing a therapeutic molecule to an animal, it does disclose in vitro transfection and does suggest to use the nucleic-acid immunoliposomes (cf. 2.1) as a DNA delivery system for humans (p.989, last paragraph + p.992 last paragraph).
A person skilled in the art would, without any doubt, follow this suggestion and administer such nuclei-acid immunoliposomes to animals/humans.

4.3. The dependent claims 34-49 do not contain additional technical features which might establish an inventive step over D1 to D7 (article 33(3) PCT). These claims are only allowable in combination with patentable independent claims.
ad claims 34-35: These claims define only usual administration methods.
ad claims 36-49: cf. points 2.1 and 2.2.

5. The subject-matter of claim 50 is not new according to Article 33(2) PCT.
For the interpretation of claim 50 confer item VIII, point 1.

5.1. The disclosure of documents D1 and D2 has already been discussed under item V, point 2.1. The presence of a container is implicit.

5.2. Furthermore, cationic immunoliposomes are disclosed in document D5. D5 discloses cationic immunoliposomes comprising lipid modified single chain anti-IgG and anti-CD fragments in a protein:lipid ratio (w:w) of about 1:20 (p. 233, point 2.5.). The immunoliposomes are suspended in an aqueous HEPES buffer.

5.3. The dependent claims 51-55 do not contain any features which, in combination with the features of any claim to which they refer, might establish novelty and an

inventive step over D1-D7 (Articles 33(2) and 33(3) PCT). The disclosures of these documents has already been discussed under points 2.1, 2.2, 5.1 and 5.2. These claims are only allowable in combination with patentable independent claims.

6.1. Document D2, which is considered to represent the most relevant state of the art, discloses (cf. p.984, first paragraph) a method for the preparation of nucleic acid-immunoliposomes from which the subject-matter of claim 13 differs in that a different process in defined.

The subject-matter of claim 13 is therefore novel (Article 33(2) PCT).

6.2. The problem to be solved by the present invention may therefore be regarded as: How to provide a process for the preparation of nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

6.3. The solution to this problem proposed in claim 13 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
None of the documents cited in the search report suggests to reverse the preparation steps (i.e. incubation of liposome with nucleic acid prior to the attachment of the antibody/antibody fragment). Furthermore, the combination of wt p53 with an antibody/antibody fragment capable of binding to a transferrin receptor has not been suggested.

6.4. Claims 14-22 are dependent on claim 13 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

7.1. Document D1, which is considered to represent the most relevant state of the art, discloses (cf. p.130, last paragraph) a method for the preparation of nucleic acid-cationic immunoliposomes from which the subject-matter of claim 23 differs in that a different antibody/antibody fragment and a different nucleic acid is used.

The subject-matter of claim 23 is therefore novel (Article 33(2) PCT).

7.2. The problem to be solved by the present invention may therefore be regarded as: How to provide a process for the preparation of nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

7.3. The solution to this problem proposed in claim 23 of the present application is considered as involving an inventive step (Article 33(3) PCT) for the following reasons:
Although the method of preparation has already been described in principal by D1, there is no suggestion in the documents cited by the search report to prepare nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.
Document D3 suggests the use of wt p53 in gene therapy in combination with transferrin. The use of an antibody fragment capable of binding to a transferrin receptor is suggested in D4 (p.10148, last paragraph) only in connection with retroviral vectors and not in connection with liposomes.
Therefore, there are no directions for a person skilled in the art to prepare nucleic acid-cationic immunoliposome complexes comprising wt p53 and an antibody/antibody fragment capable of binding to a transferrin receptor.

7.4. Claims 24-32 are dependent on claim 23 and as such also meet the requirements of the PCT with respect to novelty and inventive step.

8. None of the documents cited in the search report discloses or even suggests a kit comprising two separate containers, one comprising a cationic immunoliposome, the other a nucleic acid as defined in claim 56.

9. For the assessment of the present claims 33-49 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII

Certain observations on the international application

1. The subject-matter of present claim 50 is not clear (Article 6 PCT). A kit is generally defined by comprising at least two parts that can be considered to represent two distinct entities, e.g. a packaging unit and its contents or two different, physically separate, solutions.
   Present claim 50 defines only one of the at least two parts (a cationic immunoliposome). A second part has not been defined, and therefore does not present any restriction. At present, the second part is taken to be any container that can contain the cationic immunoliposomes.

2. The vague and imprecise statement in the description on page 26, last paragraph, implies that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity (Article 6 PCT) when used to interpret them (see also the PCT Guidelines, III-4.3a).

3. The description is not in accordance with the claims (Article 6 PCT).

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT OR THE DECLARATION

(PCT Rule 44.1)

To:
ROTHWELL, FIGG, ERNST & KURZ
Attn. SAXE, S.
Columbia Square, Suite 701 East
555 Thirtennth St., NW
Washington, D.C. 20004
UNITED STATES OF AMERICA

Date of mailing *(day/month/year)*	14/09/2000
Applicant's or agent's file reference: 2444-105.PCT	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No.: PCT/US 00/ 04392	International filing date *(day/month/year)* 22/02/2000
Applicant: GEORGETOWN UNIVERSITY et al.	

1. [X] The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Fascimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. [ ] The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. [ ] **With regard to the protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

[ ] the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

[ ] no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after **18 months** from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90*bis*.1 and 90*bis*.3, respectively, before the completion of the technical preparations for international publication.

Within **19 months** from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within **20 months** from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Catherine Humbert

Form PCT/ISA/220 (July 1998)

NOTES TO FORM PCT/ISA/220

These Notes are intended to give the basic instructions concerning the filing of amendments under article 19. The Notes are based on the requirements of the Patent Cooperation Treaty, the Regulations and the Administrative Instructions under that Treaty. In case of discrepancy between these Notes and those requirements, the latter are applicable. For more detailed information, see also the PCT Applicant's Guide, a publication of WIPO.

In these Notes, "Article", "Rule", and "Section" refer to the provisions of the PCT, the PCT Regulations and the PCT Administrative Instructions respectively.

INSTRUCTIONS CONCERNING AMENDMENTS UNDER ARTICLE 19

The applicant has, after having received the international search report, one opportunity to amend the claims of the international application. It should however be emphasized that, since all parts of the international application (claims, description and drawings) may be amended during the international preliminary examination procedure, there is usually no need to file amendments of the claims under Article 19 except where, e.g. the applicant wants the latter to be published for the purposes of provisional protection or has another reason for amending the claims before international publication. Furthermore, it should be emphasized that provisional protection is available in some States only.

What parts of the international application may be amended?

Under Article 19, only the claims may be amended.

During the international phase, the claims may also be amended (or further amended) under Article 34 before the International Preliminary Examining Authority. The description and drawings may only be amended under Article 34 before the International Examining Authority.

Upon entry into the national phase, all parts of the international application may be amended under Article 28 or, where applicable, Article 41.

When?

Within 2 months from the date of transmittal of the international search report or 16 months from the priority date, whichever time limit expires later. It should be noted, however, that the amendments will be considered as having been received on time if they are received by the International Bureau after the expiration of the applicable time limit but before the completion of the technical preparations for international publication (Rule 46.1).

Where not to file the amendments?

The amendments may only be filed with the International Bureau and not with the receiving Office or the International Searching Authority (Rule 46.2).

Where a demand for international preliminary examination has been/is filed, see below.

How?

Either by cancelling one or more entire claims, by adding one or more new claims or by amending the text of one or more of the claims as filed.

A replacement sheet must be submitted for each sheet of the claims which, on account of an amendment or amendments, differs from the sheet originally filed.

All the claims appearing on a replacement sheet must be numbered in Arabic numerals. Where a claim is cancelled, no renumbering of the other claims is required. In all cases where claims are renumbered, they must be renumbered consecutively (Administrative Instructions, Section 205(b)).

The amendments must be made in the language in which the international application is to be published.

What documents must/may accompany the amendments?

Letter (Section 205(b)):

The amendments must be submitted with a letter.

The letter will not be published with the international application and the amended claims. It should not be confused with the "Statement under Article 19(1)" (see below, under "Statement under Article 19(1)").

The letter must be in English or French, at the choice of the applicant. However, if the language of the international application is English, the letter must be in English; if the language of the international application is French, the letter must be in French.

BNSDOCID: <XSISA220NOENP4_1_>

The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped),whether

(i) the claim is unchanged;

(ii) the claim is cancelled;

(iii) the claim is new;

(iv) the claim replaces one or more claims as filed;

(v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."

2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."

3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."

4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international appplication is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

BNSDOCID: <XSISA220NOENP4_1_>

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
2444-105.PCT		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 00/ 04392	22/02/2000	22/02/1999
Applicant		
GEORGETOWN UNIVERSITY et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[X] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[ ] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [X] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. _____

[ ] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/04392

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127 C12N15/88 A61K47/48 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	COMPAGNON B ET AL: "ENHANCED GENE DELIVERY AND EXPRESSION IN HUMAN HEPATOCELLULAR CARCINOMA CELLS BY CATIONIC IMMUNOLIPOSOMES" JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 7, no. 1, 1997, pages 127-141, XP000682912 ISSN: 0898-2104 the whole document --- -/--	1-57

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 September 2000	14/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Niemann, F

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YOSHIDA J ET AL: "SIMPLE PREPARATION AND CHARACTERIZATION OF CATIONIC LIPOSOMES ASSOCIATED WITH A MONOCLONAL ANTIBODY AGAINST GLIOMA-ASSOCIATED ANTIGEN (IMMUNOLIPOSOMES)" JOURNAL OF LIPOSOME RESEARCH,US,MARCEL DEKKER, NEW YORK, vol. 5, no. 4, 1995, pages 981-995, XP000558625 ISSN: 0898-2104 the whole document ---	1-57
A	XU L ET AL: "Transferrin-liposome-mediated p53 sensitization of squamous cell carcinoma of the head and neck to radiation in vitro" HUMAN GENE THERAPY,XX,XX, vol. 8, no. 4, 1 March 1997 (1997-03-01), pages 467-475, XP002098089 ISSN: 1043-0342 cited in the application the whole document ---	4,13,23, 39,48,49
A	JIANG AN ET AL: "Cell-type-specific gene transfer into human cells with retroviral vectors that display single-chain antibodies." JOURNAL OF VIROLOGY, vol. 72, no. 12, December 1998 (1998-12), pages 10148-10156, XP002103152 ISSN: 0022-538X cited in the application the whole document ---	2,9,13, 22,23, 32,36, 37,50,51
A	J DE KRUIF ET AL: "Biosynthetically lipid-modified human scFv fragments from phage display libraries as targeting molecules for immunoliposomes" FEBS LETTERS,NL,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, vol. 3, no. 399, 16 December 1996 (1996-12-16), pages 232-236, XP002075140 ISSN: 0014-5793 the whole document --- -/--	5,9,14, 22,24, 32,37, 40,51,52

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOBATAKE E ET AL: "A FLUOROIMMUNOASSAY BASED ON IMMUNOLIPOSOMES CONTAINING GENETICALLYENGINEERED LIPID-TAGGED ANTIBODY" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 69, no. 7, 1 April 1997 (1997-04-01), pages 1295-1298, XP000689729 ISSN: 0003-2700 abstract	5,9,14, 22,24, 32,37, 40,51,52
A	WO 83 02069 A (UNIV CALIFORNIA) 23 June 1983 (1983-06-23) the whole document	6-8, 15-18, 25-28, 41-44,53

INTERNATIONAL SEARCH REPORT

national application No.
PCT/US 00/04392

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 33-49 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 00/04392

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8302069 A	23-06-1983	US 4429008 A	31-01-1984
		CA 1197190 A	26-11-1985
		EP 0097691 A	11-01-1984

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
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in its capacity as elected Office

Date of mailing (day/month/year) 21 December 2000 (21.12.00)	
International application No. PCT/US00/04392	**Applicant's or agent's file reference** 2444-105.PCT
International filing date (day/month/year) 22 February 2000 (22.02.00)	**Priority date** (day/month/year) 22 February 1999 (22.02.99)
Applicant XU, Liang et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

20 September 2000 (20.09.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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Authorized officer

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WO 00/50008 A3

(54) Title: ANTIBODY FRAGMENT-TARGETED IMMUNOLIPOSOMES FOR SYSTEMIC GENE DELIVERY

(57) Abstract: Nucleic acid-immunoliposome compositions useful as therapeutic agents are disclosed. These compositions preferably comprise (i) cationic liposomes, (ii) a single chain antibody fragment which binds to a transferrin receptor, and (iii) a nucleic acid encoding a wild type p53. These compositions target cells which express transferrin receptors, e.g., cancer cells. These compositions can be used therapeutically to treat persons or animals who have cancer, e.g., head and neck cancer, breast cancer or prostate cancer.

WHAT IS CLAIMED IS:

1. An immunoliposome comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid.

2. The immunoliposome of claim 1 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

3. The immunoliposome of claim 1 wherein said nucleic acid is DNA.

4. The immunoliposome of claim 1 wherein said nucleic acid encodes a wild type p53.

5. The immunoliposome of claim 1 wherein said antibody or antibody fragment comprises a lipid tag.

6. The immunoliposome of claim 1 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a sulfhydryl group at a carboxy terminus on said antibody or antibody fragment.

7. The immunoliposome of claim 6 wherein said sulfur atom is part of a cysteine residue.

8. The immunoliposome of claim 6 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

9. The immunoliposome of claim 1 wherein said antibody fragment is a single chain.

10. The immunoliposome of claim 1 wherein said antibody or antibody fragment and said cationic liposome are present at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

11. The immunoliposome of claim 1 wherein said nucleic acid and said cationic liposome are present at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

12. A pharmaceutical composition comprising the immunoliposome of claim 1.

13. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) mixing nucleic acid encoding a wild type p53 with a cationic liposome to produce a nucleic acid-liposome complex;

b) preparing an antibody or antibody fragment capable of binding to a transferrin receptor; and

c) mixing said nucleic acid-liposome complex with said antibody or antibody fragment to form said nucleic acid-cationic immunoliposome complex.

14. The method of claim 13 wherein said antibody or antibody fragment comprises a lipid tag.

15. The method of claim 13 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

16. The method of claim 15 wherein said reducible group is a sulfhydryl.

17. The method of claim 16 wherein said sulfhydryl is part of a cysteine residue.

18. The method of claim 15 wherein said cationic liposome comprises DOPE linked to MPB or other sulfhydryl reacting group.

19. The method of claim 13 wherein said nucleic acid is DNA.

20. The method of claim 13 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

21. The method of claim 13 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

22. The method of claim 13 wherein said antibody fragment is a single chain.

23. A method of preparing a nucleic acid-cationic immunoliposome complex comprising the steps of:

a) preparing an antibody or antibody fragment capable of binding to a transferrin receptor;

b) mixing said antibody or antibody fragment with a cationic liposome to form a cationic immunoliposome; and

c) mixing said cationic immunoliposome with nucleic acid encoding a wild type p53 to form said nucleic acid-cationic immunoliposome complex.

24. The method of claim 23 wherein said antibody or antibody fragment comprises a lipid tag.

25. The method of claim 23 wherein said antibody or antibody fragment comprises a reducible group at a carboxy terminus prior to mixing with said nucleic acid-liposome complex.

26. The method of claim 25 wherein said reducible group is a sulfhydryl.

27. The method of claim 26 wherein said sulfhydryl is part of a cysteine residue.

28 The method of claim 25 wherein said cationic liposome comprises MPB-DOPE.

29. The method of claim 23 wherein said nucleic acid is DNA.

30. The method of claim 23 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

31. The method of claim 23 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

32. The method of claim 23 wherein said antibody fragment is a single chain.

33. A method for providing a therapeutic molecule to an animal in need thereof, comprising administering to said animal a therapeutically effective amount of a nucleic acid-cationic immunoliposome complex comprising i) a cationic liposome, ii) an antibody or antibody fragment, and iii) a nucleic acid.

34. The method of claim 33 wherein said complex is administered systemically.

35. The method of claim 33 wherein said complex is administered intravenously.

36. The method of claim 33 wherein said antibody or antibody fragment is capable of binding to a transferrin receptor.

37. The method of claim 33 wherein said antibody fragment is a single chain.

38. The method of claim 33 wherein said nucleic acid is DNA.

39. The method of claim 33 wherein said nucleic acid encodes a wild type p53.

40. The method of claim 33 wherein said antibody or antibody fragment comprises a lipid tag.

41. The method of claim 33 wherein said antibody or antibody fragment is covalently bound to said cationic liposome via a sulfur atom which was part of a reducible group at a carboxy terminus on said antibody or antibody fragment.

42. The method of claim 41 wherein said reducible group is a sulfhydryl.

43. The method of claim 42 wherein said sulfhydryl is part of a cysteine residue.

44. The method of claim 41 wherein said antibody or antibody fragment is covalently bound to DOPE linked to MPB or other sulfhydryl reacting group.

45. The method according to claim 33 wherein said antibody or antibody fragment and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

46. The method according to claim 33 wherein said nucleic acid and said cationic liposome are present in said nucleic acid-cationic immunoliposome complex at a nucleic acid:lipid (μg:nmol) ratio in the range of 1:6 to 1:20.

47. The method according to claim 33 wherein said animal is a human.

48. The method according to claim 33 wherein said animal has cancer.

49. The method according to claim 48 wherein said cancer is selected from the group consisting of i) head and neck cancer, ii) breast cancer and iii) prostate cancer.

50. A kit comprising a cationic immunoliposome wherein said cationic immunoliposome comprises a transferrin receptor binding antibody fragment.

51. The kit of claim 50 wherein said antibody fragment is a single chain.

52. The kit of claim 50 wherein said antibody fragment comprises a lipid tag.

53. The kit of claim 50 wherein said antibody fragment is conjugated to a cationic liposome.

54. The kit of claim 50 said antibody fragment and cationic lipids are present in a protein:lipid ratio (w:w) in the range of 1:5 to 1:40.

55. The kit of claim 50 wherein said cationic immunoliposome is in an aqueous solution.

56. The kit of claim 50 further comprising a nucleic acid for use as a positive control in a container separate from said cationic immunoliposome.

57. The kit of claim 56 wherein said nucleic acid encodes a reporter gene selected from the group consisting of luciferase, β-galactosidase and green fluorescent protein.